Studies of leaf surface fungi in relation to flavour of tea

U. K. K. Wickremasinghe

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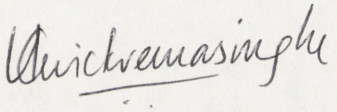
Declaration

Except where otherwise indicated
this thesis is my own work.



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Abstract

Tea is a widely consumed beverage that constitutes an important internationally traded commodity. The appearance, particle size and the quantity of tea available are important determinants of market price of tea. However, the unit price of tea is determined to a large degree by its flavour. Flavour is a term collectively used to describe the sensory properties 'taste' and 'aroma' of brewed tea. While the taste characterises tea as a beverage, the aroma distinguishes flavoury teas from ordinary teas. A price differential of as much as ten times is sometimes observed between the seasonal flavoury teas and the 'plain' teas abundantly found in the market place. Flavour is therefore of immense economic significance for the tea producing countries whose economic fortunes depend on earnings accrued by the tea trade. However, its seasonal, ephemeral nature imposes limits to full realization of the economic advantage of flavour. Outstanding flavour of tea in Sri Lanka is confined to two brief, well defined seasons: January and February in Dimbula District and July-September in Uva district. Achieving control over the process of flavour synthesis in tea is an attractive proposition due to the economic rewards this phenomenon brings.

Lack of sound explanation of the phenomenon of seasonal tea flavour has aborted attempts to induce flavour. In the absence of an explanatory hypothesis for tea flavour, tea researchers have retreated to consider its development as an intractable natural phenomenon. Tea industry sources in Sri Lanka believe that a 'pink yeast' colonizing tea leaf surfaces during the flavour season is partly or wholly responsible for the development of flavour. Based on this belief it is postulated here that the pink yeast *Rhodotorula glutinis* enriches the tea aroma complex by supplementing the carotenoids content in the tea leaf during the flavour season. Thus the underlying assumption of this proposition draws from the recent reports that the carotenoids contribute to tea aroma by forming oxidation products during tea manufacture. Proof of the hypothesis depends largely on the successful colonization of the leaf surface and carotenoids biosynthesis thereon by *R. glutinis* under conditions similar to those occurring during the flavour season in Sri Lanka. A major part of this dissertation reports results of investigations into the effect of environmental factors on the growth and carotenoids production by the yeast *R. glutinis*. For the first time high pressure liquid chromatography (HPLC) has been employed in the

separation of yeast carotenoids complex providing a rapid, more sensitive and accurate technique for this purpose. High performance liquid chromatography can now replace thin layer chromatography (TLC) which, although the traditionally used method of carotenoids analysis, is particularly unsuited for the analysis of carotenoids.

Successful colonization of the green tea leaf surfaces by the yeast *R. glutinis* is an essential prerequisite for its proposed role in tea flavour development. The subject of microbial colonization of green leaf surfaces and associated phenomena e. g. population composition and its variation, microbial interactions on leaf surfaces, have been amply documented during the last three decades. With a view to gaining an impression of characteristics of phylloplane microbial populations, the phylloplane microflora of the evergreen ornamental plant *Photinia glabra* was sampled fortnightly. The phylloplane microflora obtained in culture is often limited by the composition of the culture medium. After a comparison of a number of commonly used laboratory culture media potato-dextrose-agar (PDA) was chosen as the medium that facilitated the growth of the widest range of fungi. Methods used in the study of the phylloplane have been often criticized as being inexact and non-quantitative. Therefore the study of the phylloplane was preceded by a comparison of direct and cultural methods commonly used in the study of the phylloplane. Leaf washing method, washed leaf prints and a leaf print technique were employed as cultural techniques while direct examination of the leaf surface was carried out with scanning electron microscopy. Whereas leaf prints isolated the highest number of fungi and yeasts, the leaf washing method provided the lowest estimates. In the case of the yeasts the relative efficiencies of the three techniques differred only marginally. Estimates provided by the washed leaf print method approximately accounted for the difference between the washing and leaf print techniques. The estimates provided by different methods on the same sampling date lacked aggreement. Large values of standard deviation and standard error were characteristic of the data yielded by the cultural techniques, particularly in the case of yeasts. Increases in sample size within the manageable range did not reduce the values of these parameters. The microbial population on *P. glabra* was dominated by the fungi *Epicoccum nigrum, Cladosporium cladosporioides* and *Alternaria alternata* and the yeasts *R. glutinis* and *Cryptococcus albidus*. Numerous other species of filamentous fungi belonging to Fungi Imperfecti were isolated infrequently. Disparities between data provided by the direct and cultural techniques indicate the need to complement data obtained by any one method.

Highly significant ($P<0.05$) differences between the adaxial and abaxial surface

populations of *E. nigrum* and *C. cladosporioides* were observed. Cultural methods consistently reported higher levels of fungal populations on the adaxial surface. The yeast populations did not exhibit such differences between the two surfaces. However, direct observations with the scanning electron microscope (SEM) revealed that many of the propagules on the adaxial surface were in an inactive state. Fungal activity was largely confined to the abaxial leaf surface. Germinated spores or mycelial growth on the adaxial surface was of rare occurrence. Substantial mycelial growth could be observed on the abaxial surface, particularly in the vicinity of stomata. No sporulating fungal colonies were observed at any stage. Results yielded by the cultural techniques highlighted the risks involved in employing only these methods in the studies of the phylloplane.

The environmental and other factors that govern variations in leaf surface microbial populations have not been elucidated previously. Such factors of the environment which govern population levels of microorganisms are of importance in explaining the seasonality of growth of yeasts on tea leaf surfaces. Earlier attempts to correlate leaf surface microbial population levels with weather factors immediately prior to sampling have been largely unsuccessful. An attempt was made here to correlate more remote meteorological data with phylloplane microbial population levels. The total microbial population and the population of *E. nigrum* showed high correlation with total rainfall, average relative humidity and the difference between maximum and minimum temperatures for the previous fortnight prior to sampling date.

The governing effect of the environmental factors on the population levels and composition of the leaf surface microflora point to major differences between the microflora on *P. glabra* in Canberra and tea in Sri Lanka. Tea cultivation has begun recently in tropical north Queensland. This provided an opportunity of studying the phylloplane microflora of tea under similar environmental conditions to Sri Lanka. The aerial microflora and the tea leaf surface microflora were studied in two subsequent years (1985 and 1986) during the 'dry season' in north Queensland. It was observed that the composition of the leaf surface microflora closely resembled the aerial microflora. Even though the aerial fungal flora exhibited differences in composition, the yeast flora under tropical and mild temperate conditions were similar, consisting mainly of the species *Rhodotorula, Sporobolomyces* and *Cryptococcus*.

Apart from the environmental factors that influence the size of the aerial microflora, the availability of water is generally considered the factor which limits microbial colonization

of green leaf surfaces. Relatively few studies of water relations of phylloplane fungi have been conducted and most are flawed by deficiencies in the experimental systems employed. The effectsof osmotic water potential and temperature on spore germination and radial mycelial growth of three common phylloplane fungi were studied using four ionic and nonionic solutes to obtain nine water potentials (-1 to -14 MPa) of a minimum nutrient culture medium. The cultures at each water potential were incubated at three temperatures (10°C, 15°C and 25°C). On the unamended culture medium, germ tube initiation took place in less than 6 h. This duration increased progressively with decreasing water potential beyond -2 MPa. At moderately low water potentials (-2 to -4 MPa) germination took place after 12 h. The spores of *A. alternata* were an exception to this trend by germinating in less than 6 h at -5 MPa. The improvement of the rate of spore germination observed with time at low water potentials was marginal and varied with the fungus. Mycelial growth of all fungi was stimulated by moderately low water potentials (-2 to -3 MPa), but declined progressively with further lowering of water potentials. Measurable growth ceased at -8 to -10 MPa.

The climatic conditions under which tea flavour biogenesis occurs in Sri Lanka are characterised by the lack of rainfall, dry windy days and cold nights and, moderately high temperatures. Under such conditions the leaf water potential could be expected to decrease causing water stress in phylloplane microorganisms. The capacity of the yeast *R. glutinis* to maintain growth and synthesize carotenoids was tested under similar laboratory conditions of temperature and water potential. At lower temperatures (5°C and 10 °C) a higher proportion of the carotenoids complex consisted of carotenoids other than torularhodin while this trend reversed at higher temperatures (30°C). The growth of the yeast continued until -14 MPa water potential though at progressively decreasing rates, particularly below -7 MPa. The water potential, the solute used to adjust water potential and the temperature of incubation exhibited strong interactions. The yeast tolerated lower water potentials closer to the optimum temperature for growth. The water potential at which carotenoids biosynthesis ceased was higher than that at which the cessation of growth occurred. The solutes exerted a selective effect on biosynthesis of carotenoids. Higher levels of carotenoids biosynthesis were observed in KCl amended media than in sucrose amended media. The age of culture had a significant effect on both the quality and quantity of the carotenoids biosynthesized. A large increase in carotenoids biosynthesis, though not in torularhodin content, was observed from the third to the fifth day after inoculation. The total carotenoids content exhibited only a marginal increase beyond the seventh day

whereas torularhodin content increased steadily. Illumination during the initial 6 h of culture appeared to have a significant effect on carotenoids biosynthesis. The biosynthesis of carotenoids continued irrespective of the subsequent conditions of illumination.

The comparatively recent separation method high pressure liquid chromatography (HPLC) appears to be a suitable technique for the analysis of yeast carotenoids. This rapid, efficient, sensitive and versatile technique has found application in the analysis of numerous biochemical compounds from a variety of other substrates. Reversed phase HPLC has been recommended for the analysis of carotenoids. Chapter 6 introduces the general principles and main forms of chromatography. Particular attention has been paid to thin layer chromatography (TLC), the traditionally employed form of chromatography in the analysis of carotenoids. It is argued that the chemical structure of the carotenoids makes them unsuitable for separation by TLC. These compounds lend themselves to structural transformation and their recovery is often incomplete. High pressure liquid chromatography however, provides a modern, rapid, accurate method of carotenoids analysis.

Carotenoids complex biosynthesized by the yeast *R. glutinis* was separated using HPLC in order to evaluate the effect of environmental conditions on β-carotene production. The HPLC column used (Alltech C18 column) was characterized in terms of number of theoretical plates and retention time of synthetic β-carotene standards. Gradient elution with 2-propanol and acetonitrile-water provided short retention times and satisfactory resolution. Increasing the percentage content of 2-propanol in the gradient helped shorten the retention times. But higher contents of 2-propanol eluted the xanthophyll torularhodin without adequate retention. Solvent gradient consisting of (10%) 2-propanol afforded optimum retention times and separation of the xanthophylls. The standard conditions of culturing the yeast *R. glutinis* (incubation on PDA for 3 d at 25°C) seemed to favour the biosynthesis of β-carotene. Under these conditions β-carotene constituted approximately 45% of the carotenoids complex as determined by HPLC. The age of culture and temperature of incubation seemed to exert profound qualitative and quantitative effects on the biosynthesis of carotenoids. Increasing age of culture favoured the biosynthesis of torularhodin in preference to β-carotene. Increasing temperature exhibited a similar effect. These observations support the biosynthetic pathway proposed by Simpson (1972). According to this pathway, the intermediate compound γ-carotene is converted to either β-carotene or torularhodin in a temperature sensitive reaction. The increased availability of carbon alone, and carbon in association with nitrogen increased the biosynthesis of the

total carotenoids complex and β-carotene. Nitrogen alone failed to induce such increases. These results suggest that the process of growth receives priority over the biosynthesis of carotenoids, which accelerates upon the completion of growth. The effects of illumination on the biosynthesis of β-carotene are similar to the effects on the total carotenoids, with a dependance on illumination for the initiation of the process.

The carotenoids content of tea leaves exposed to different environmental conditions were analysed using HPLC. The HPLC conditions determined were suitable for the separation of tea leaf carotenoids. An analysis of the carotenoids content of the bud, the first, second and third leaves revealed that the carotenoids content decreases with increasing age of the leaf. The chlorophyll content exhibited an increase with leaf age. The carotenoids consisted predominantly of β-carotene and lutein while violaxanthin and neoxanthin were present in substantial quantities. Increased light intensity seemed to increase the carotenoids content of the tea leaf. An accompanying increase in the chlorophyll content was observed. The considerable difference between the daily maximum and minimum temperatures stimulated the biosynthesis of β-carotene. The above observations lead to the conclusion that the environmental conditions during the flavour season in Sri Lanka increase the biosynthesis of carotenoids in the tea leaf. These observations are supportive of the hypothesis propounded by Wickremasinghe (1974).

Studies reported here support the hypothesis that the yeast *R. glutinis* enriches tea flavour by complementing the carotene content of the tea leaf during the flavour season. The increase in leaf surface yeast populations and the amount of carotenoids biosynthesized under limited availability of moisture and high temperatures indicate that the proposed flavour enrichment is a distinct possibility. Actual proof however, awaits inoculation of the green tea leaf with the yeast and the analysis of aroma complex of the teas brewed from the processed leaf. It appears that for the first time this yeast offers an opportunity of inducing tea flavour.

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Chapter 1

General Introduction

1.1. Microbiology of the phylloplane

Study of saprophytic microflora colonizing green leaf surfaces is of comparatively recent origin. Despite early reference to leaf surface fungi (de Bary, 1866), the existence of a characteristic microflora on aerial plant surfaces had not been propounded until the discovery of a large air spora, enabled by more efficient trapping devices (Gregory and Hirst, 1957). This development highlighted that the propagules of plant pathogenic fungi constituted a fraction of the total air microflora, which was augmented by a diverse array of saprophytic fungi. Predominant among such saprophytic species are the members of the family Sporobolomycetaceae and *Cladosporium* species. Plant pathologists pioneering investigations into the influence these fungi might have on the development of foliar diseases found a new dimension to the host-pathogen-environment triangle. However, the early research work was typified by its tendency to regard the leaf surface as an identical environment to the then familiar rhizosphere. This attitude was given expression when the term 'phyllosphere' was coined by Last (1955) and Ruinen (1956) to describe the habitat on leaf surfaces. Differences in respective substrates and the surrounding medium were later recognized by renaming this habitat 'phylloplane' (Kerling, 1958).

Since research on the microbiology of the phylloplane began, many workers have studied the phylloplane microflora of different plant species. These studies concentrate on enumeration of the fungal flora (Diem, 1974; Bernstein and Carroll, 1977 ; McBride and Hayes, 1977) and fungal successions (Hogg and Hudson, 1966; Dickinson, 1967; Lindsey and Pugh, 1976; Mishra and Dickinson, 1981; Mishra and Dickinson, 1984) on leaf surfaces. Such studies have been motivated by the need to distinguish between the 'resident' and the 'casual' microflora on leaf surfaces and to determine their origins. However the results do not point to any fungal species preferentially or exclusively colonizing leaf surfaces. The numerically important fungi on leaf surfaces appear to be reflecting their abundance in the atmosphere and thus better access to leaf surfaces. Success upon deposition seems to depend on environmental factors rather than any unique

characteristic associated with the leaf surface *per se*. Investigations on fungal successions however, are more enlightening. Present knowledge, that fungal colonization of green leaves begins at an early age and that the fungal activity accelerates at the onset of leaf senescence, emanates from the work in this area. Studies involving analyses of microbial populations on leaf surfaces in the northern and southern hemispheres reveal a general pattern of colonization. Surfaces of emerging and very young leaves are colonized predominantly by bacteria and yeast-like fungi of the families Cryptococcaceae, Sporobolomycetaceae, and of the genus *Aureobasidium* (Pugh *et al.*, 1972). The bacterial numbers usually increase significantly before yeasts and filamentous fungi colonize green leaves (Ruinen, 1961).

1.1.1. Fungi on leaf surfaces

Three decades of research on the microbiology of the phylloplane reveal some common features of the fungal flora inhabiting the leaf surface environment and its patterns of colonization. The majority of the more than 100 fungal taxa which have been reported on green leaf surfaces individually constitutes less than five per cent of the populations. Further, the studies on temperate and tropical plant species show that substantial colonization of the leaf surface by *Cladosporium* spp. is a universal phenomenon. Proof that many of the regularly reported fungi grow continuously on green leaf surfaces is lacking. Ruscoe (1971) estimated that fungi occupied from less than 1-10% of the surfaces of *Nothofagus* leaves, with the proportion increasing as the leaves aged. Diem (1974) reported that the area occupied by fungi did not exceed two per cent of the leaf surface. However, sporulating colonies of these fungi are common on newly-dead leaves of many plants. These phenomena suggest that the leaf surface acts as a trap for many fungal propagules in the air spora without necessarily providing a favourable environment for subsequent germination and growth. Presumably, the fungi on leaf surfaces exist as vegetative structures alternating between growth and survival rather than continuously growing mycelia. The evident success of *Cladosporium* spp. is attributable to its capacity to form microsclerotia which are resistant to desiccation (Pugh and Buckley, 1971 ; Skidmore, 1976). Several other species of fungi develop pigmented, suberized hyphae or adopt a yeast-like growth pattern by forming secondary spores which ensure survival during adverse conditions (Skidmore, 1976). The most successful among the yeasts, *Sporobolomyces* spp., exhibits capacity to commence growth quickly once favourable conditions recur. The rapid development of most phylloplane fungi awaits erosion of

epicuticular waxes, damage to cuticle or alterations in the host physiology (Dickinson and Wallace, 1976). Studies on the distribution of fungi on leaf surfaces have highlighted the differences between the upper and lower surface populations (Ruscoe, 1971).

1.1.2. Yeasts on the phylloplane

Presence of yeasts on leaf surfaces is likely to be underestimated due to lack of cell motility and relatively low yeast cell concentrations found in air microflora. Numerous reports of abundant leaf surface yeast populations (Last, 1955; di Menna, 1956; Pennycook and Newhook, 1981) suggest that contamination with soil (a rich source of yeasts) during germination, and insect transmission of yeasts provide adequate inoculum. The members of the Cryptococcaceae and Sporobolomycetaceae are the commonest microorganisms on leaf surfaces (Last and Warren, 1972). This suggests that the yeasts are among the most successful colonizers of the phylloplane. The population estimates obtained by cultural methods of study indicate that the numbers of yeast-like forms usually exceed those of filamentous fungi. Among the yeasts, the members of the Cryptococcaceae (e.g. *Rhodotorula, Cryptococcus, Torulopsis*) and Sporobolomycetaceae (e.g. *Sporobolomyces* and *Tilletiopsis*) and the genus *Aureobasidium* predominate to the virtual exclusion of the yeasts of the family *Endomycetaceae* (e.g. *Saccharomyces, Hanseniospora*). Seasonal variations in yeast populations have been reported (di Menna, 1958). Hislop and Cox (1969) reported a summer increase in pink yeast populations.

1.1.3. Tea culture

The tea plant is a member of the family Theaceae and is botanically named *Camellia sinensis* (L.) O. Kuntze. Tea is believed to have originated in east Asia. In its natural habitats, tea grows to a height of more than 3 m and develops a defined stem. Regular prunning carried out under cultivation, imparts a bushy growth habit and allows only a maximum height of 1 m. Long, elliptical tea leaves are pubescent along the underside of the mid rib. The upper side is shiny and the edges are serrated. Due to its out breeding nature, tea is liable to extensive phenotypic variation in growth, yield and organoleptic properties of made tea. Propagation is generally carried out vegetatively by means of cuttings consisting of a single leaf, an auxilliary bud and a shoot segment of 2.5 cm. Cuttings are obtained from bushes selected on the basis of beverage quality, yield and resistance to pests and diseases. These cuttings are raised in nurseries (in polythene bags) under intensive care for 6-9 months and then transplanted in the field. Satisfactory growth

of the tea plant requires adequate rainfall, good drainage and slightly acidic soils. Many tropical and semitropical areas in the world thus become suitable for tea cultivation. The plant which achieves it maximum yield potential around 10 y of age, remains productive for many decades. The apical bud and the two leaves below it constitute the economic yield of tea and is referred to as the 'flush' in tea jargon. Harvesting is done at intervals of 6-10 d and the harvested leaf is processed in less than 24 h. Tea is produced by a number of developing countries in Asia and Africa and consumed mainly in Britain, West Germany and The Netherlands.

1.1.4. Tea in Sri Lanka

Tea is synonymous with Sri Lanka. Since the introduction of the tea plant (following the devastation of its coffee industry by the 'coffee rust' caused by the fungus *Hemileia vastatrix*), tea cultivation spread across the landscape of the island to occupy more than 240 000 ha at present. These estates range in size from less than 2 ha peasant holdings to 1 000 ha plantations, and occur at elevations ranging from sea level to 2 000 m. These produce around 210 Mkg of black tea annually, gaining the position for Sri Lanka of the third largest tea producer in the world. Teas originating from higher altitudes in general are reputed for better quality whereas those produced in lower altitudes possess more colour and strength. Tea occupies an extremely important place in the island's economy by accounting for more than 40% of the foreign exchange earnings. In addition, the tea sector employs nearly 2 million people, and is the largest employer in the country. Thus, the country's fortunes are closely linked to the increased earnings of the tea industry.

1.2. Flavour in tea

1.2.1. The tea manufacturing process

The tea manufacturing process (conversion of green tea leaf into black tea) is a mechanical process pursued in order to facilitate a series of complex biochemical reactions. These reactions result in chemical complexes that impart distinct characteristics to commercial black tea. Following a period of preconditioning the chemical compounds compartmentalized in different cellular and intercellular regions of the leaf are allowed to mix by the deliberate disruption of cells. The manufacturing process consists of a) withering, b) rolling (leaf maceration), c) fermentation, d) firing (drying), and e) sorting and packaging. The entire process is usually completed in less than 24 h. It is only the

rapidly growing bud and the two youngest leaves which are suitable for this process, due mainly to chemical composition. Plucking (harvesting) of the green leaf triggers off a series of chemical reactions that are similar to those accompanying leaf senescence. The changes in cell wall permeability caused by the loss of turgidity following plucking allows slow, initial mixing of chemical substances (Wickremasinghe, 1974). Thus in a strict biochemical sense, manufacture starts at plucking. However, control over the sequence of biochemical events following harvesting is not achieved until withering of the leaf begins at the factory.

Withering: The harvested leaf which may contain up to about 75% moisture in the field is spread on troughs and subjected to partial desiccation by blowing hot or cold air through until the moisture content is reduced to 55-45%. During withering the leaf undergoes physical changes in elasticity, turgour, size, weight and volume. The importance of obtaining the correct degree of wither for the success of subsequent steps of the manufacturing process cannot be overstated. Withering, which was earlier thought only to be bringing about a reduction in moisture content, has begun to command careful consideration following reports (Roberts and Sanderson, 1966) that leaf chemical constituents undergo important changes during the process. Considerable biochemical changes of the flavanoids complex, the amino acids, essential oils and the enzyme activity occurring at this stage contribute to the formation of taste and aroma during fermentation. The withering process may take 6-18 h depending on the atmospheric humidity and the quality of the final product aspired.

Rolling: The process of leaf maceration, brings the major group of chemical constituents of the tea leaf -the flavanols- into contact with the enzyme catechol oxidase. Disruption of cells is achieved by subjecting the leaf (on a horizontal metal surface) to the reciprocating action of a heavy mechanical device. The rolling process interferes with the normal metabolic process of cells and replaces it with fermentation, which is the basic process in black tea manufacture. Rolling is carried out intermittently to prevent development of a high temperature that may deactivate the enzyme. Leaf colour changes gradually from dark green to coppery red and a pleasant odour is developed during the rolling process. Severity of rolling determines particle size, a criterion used in grading, and a characteristic associated with specific quality categories. Higher quality 'tippy' teas are thus subjected to limited periods of light rolling whereas coloury 'dust' grades are rolled liberally.

Fermentation: The withering and rolling processes described above are prerequisites for

the major chemical reactions to follow. In addition, these steps in the manufacturing process are extremely important (in their own right) in the development of components of the aroma complex. The flavanols undergo atmospheric oxidation catalyzed by the enzyme catechol oxidase brought into contact during rolling. Fermentation is allowed to take place for a period ranging from 2-4 h at ambient temperature. Resultant complexes of this reaction determine to a great extent the colour and flavour of made tea.

Firing: The fermentation process is terminated by subjecting the leaf mass to a temperature of 90°C in a 20 min period. At the end of the firing, the moisture content of the tea falls to about 3%. During firing the tea leaf undergoes final physical and chemical changes that impart specific taste, colour and odour. The final weight of the manufactured tea is approximately 25% of that of the green leaf.

1.2.2. Biochemistry of tea manufacture

The major biochemical reactions occurring during tea manufacture and discussed under relevant processes are diagramatically presented in Figure 1-1. The mechanical process described in the preceding section is carefully manipulated to produce predetermined characteristics (of particle size, colour and flavour) in made tea. However, the final quality is largely determined by the chemical composition of the tea leaf which demonstrably depends on environmental factors (Ramaswamy, 1963 ; Bhatia and Ullah, 1968) and genetic constitution (Sanderson, 1964a; Bhatia and Ullah, 1968). Such variation may be responsible for the lack of conformity among published data on chemical constituents of tea leaf. However, data in Table 1-1, representing the average, have been reported by Millin and Rustidge (1967).

The major chemical component of the tea leaf, the polyphenols constitute 20-30% of the leaf dry matter. It has been suggested that the relative contents of individual polyphenols may be genetically controlled (Bhatia and Ullah, 1962). The polyphenols are biosynthesized predominantly in the immature leaves of the tea plant (Sanderson and Sivapalan, 1966). Polyphenols undergo oxidation during fermentation to form complexes that impart specific organoleptic characteristics to the made tea. The enzymic oxidation of the catechins to form highly reactive quinones constitutes the first step in tea fermentation. Tea beverage quality is positively correlated with the flavanol concentration in the green tea leaf. Theaflavins and thearubigins are formed as the main catechin oxidation products of fermentation. Theaflavins impart colour and brightness, and the ratio of theaflavins to thearubigins determines strength of the tea liquor (Roberts and Smith, 1963).

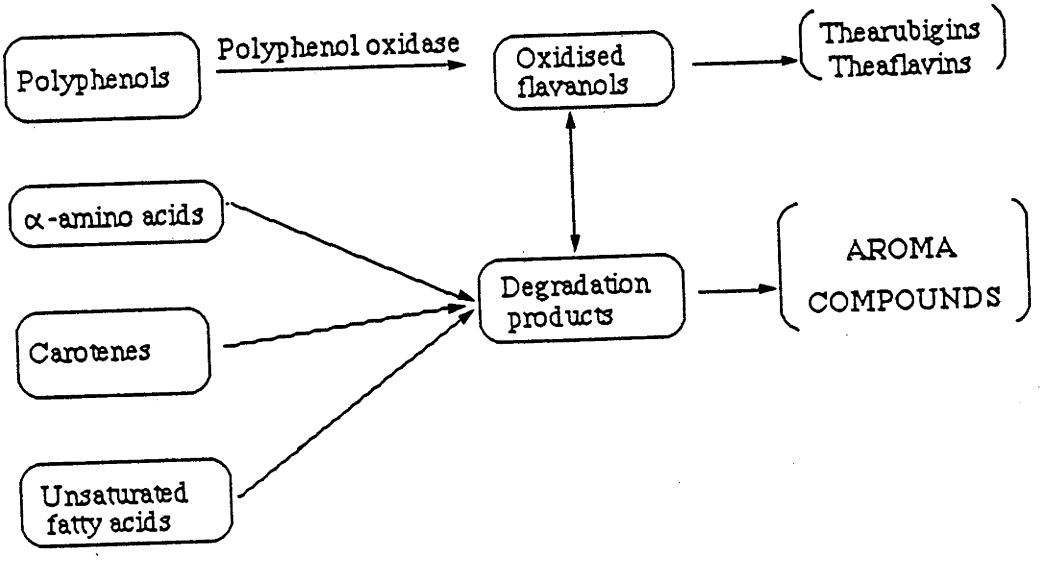


Figure 1-1: Flow chart of biochemical changes during tea manufacture

Table 1-1: Chemical composition of the tea leaf[a]

Component	Amount in flush (% dry wt.)
Substances soluble in hot water	
Flavanols	17 - 30
Flavanols and flavanol glycosides	3 - 4
Leucoanthocyanins	2 - 3
Polyphenolic acids and depsides	~ 5
Total polyphenols	~30
Caffeine	3 - 4
Amino acids	~ 4
Simple carbohydrates	~ 4
Organic acids	~ 0 . 5
Substances partially soluble in hot water	
Polysaccharides	~ 13
Proteins	~ 15
Ash	~ 5
Substances insoluble in water	
Cellulose	~ 7
Lignin	~ 6
Lipids	~ 3
Pigments	~ 0. 5
Volatile substances	0 . 01 - 0 . 02

[a] Adapted from Millin and Rustidge (1967). ~ indicates approximate values

Enzymes: Many enzymes that participate in the metabolism of leaf pigments, proteins and flavanoids have been found in the green tea leaf (Sanderson and Roberts, 1964 ; Ogura, 1969; Wickremasinghe, 1969) . Although the primary role of these enzymes is as catalysts of oxidation and reduction reactions in the tea plant, their study has been concerned mainly with tea processing. The enzyme which assumes a key role in tea manufacture is catechol oxidase. With the use of immunological techniques, Wickremasinghe *et al.* (1967) showed that this enzyme is located in the epidermis and around vascular bundles of the tea leaf. Leaf maceration in the manufacturing process is carried out to allow catechol oxidase to catalyze atmospheric oxidation of polyphenols which are localized in the vacuoles of the palisade parenchyma of tea leaf tissues.

Amino acids: Although all amino acids usually associated with proteins have been reported in tea, (E. A. H. Roberts and Wood, 1951; G. R. Roberts and Sanderson, 1966) the most abundant is theanine which is unique to tea (Sakato *et al.*, 1950). The amino acids undergo chemical changes during manufacture to result in important aroma compounds. Younger leaves have been found to contain higher levels of free amino acids (Popov, 1956).

Leaf pigments: The main pigments in fresh tea flush are chlorophylls and carotenoids (Wickremasinghe *et al.*, 1965; Co and Sanderson, 1970). During tea manufacture, a large decrease in chlorophyll content is observed with a concomitant increase in its degradation products (Wickremasinghe *et al.*, 1965). The decrease in chlorophyll content has been estimated to be about 75%. Tirimanne and Wickremasinghe (1965) enumerated 14 carotenoids in the tea leaf, identifying only nine of them. Subsequent studies (Sanderson *et al.*, 1971; Ullah, 1979) have enumerated only four carotenoids, a fact attributed to qualitative differences in materials used in these investigations. Studies of compositional changes (Hazarika and Mahanta, 1984) and degradation of carotenoids (Hazarika and Mahanta, 1983) during black tea manufacture have proved that they contribute significantly to the formation of tea aroma.

Caffeine: Tea contains 3-5% (on a dry weight basis) of caffeine (Wood *et al.*, 1964) and much smaller amounts of theobromine and theophylline. Caffeine content has been found to increase during withering (Sanderson, 1964b).

1.2.3. Flavour

Flavour is one of the subjective sensory properties of a food by which it is recognized and enjoyed. Flavour assumes special significance in the international tea trade by forming the basis of tea valuation. Although the appearance, colour, texture and temperature of a food contribute to flavour, taste and aroma are considered its major components (Moncrief, 1967). The compounds that contribute to aroma in food systems exist as trace components unlike taste compounds which exist in relative abundance. Isolation and identification of volatile aroma compounds thus become tedious. The advents of gas liquid chromatography and mass spectrophotometry have proved invaluable in the volatile aroma research. Using these techniques, approximately 5 000 volatile compounds have been identified in foods. Tea contains more than 300 identified volatiles (Yamanishi *et al.*, 1968a). The dependant relationship between tea flavour and its aroma has been demonstrated by tasting teas before and after removal of aroma. Dearomatized tea infusions are almost flavourless and only slightly astringent. Flavour production is generally sought during tea manufacture due to its economic value.

Many of the aroma compounds are generated during the processing of the food. Processing may include enzymic action, chemical reactions, auto-oxidation, microbial fermentation and cooking. Volatile aroma compounds of tea are formed during the final stage of manufacture (firing). The composition of the aroma complex however, is dependant upon precursors formed by the catabolic reactions undergone by the leaf amino acids, polysaccharides, pigments and other lipids during fermentation. These nonvolatile precursors formed during fermentation react via the Maillard reaction to form compounds responsible for the typical aroma of a tea. The chemical constitution of the leaf and the processing conditions equally influence final flavour.

Chemical compounds that contribute to tea flavour have been broadly classified into nonvolatile extractable solids that contribute to 'taste' and volatile fraction responsible for 'aroma' (Stahl, 1962). The aroma fraction constitutes less than 0.01% of the leaf dry weight. Teas from different regions possess characteristic aromas so pronounced that a professional organoleptic assessment could identify the season and the geographical origin. Appreciable quantitative differences in the total and relative contents of several aroma constituents have been observed in an analysis of black teas originated from the Dimbula and Uva districts in Sri Lanka, Nilgiri and Darjeeling districts in India and Shizuoka district in Japan (Yamanishi *et al.*, 1968a). Similar analyses of black teas from Sri Lanka

have showed that high-grown (better quality) teas contain higher quantities of volatiles than low-grown (lower quality) teas (Yamanishi *et al.*, 1968b). Varietal differences in aroma potential of the tea flush also have been reported (Takeo and Mahanta, 1983).

1.2.4. Tea aroma formation

Black teas contain a distinctive aroma from the fresh tea flush from which they originate. Many of the compounds identified in black tea aroma have not been found in fresh green tea flush. The aroma of the green tea flush changes during the manufacturing process, attaining the characteristic black tea aroma after the final (firing) stage of manufacture. Fresh tea leaf contains more alcohols whereas manufactured black tea contains more aldehydes and acids (Saijo and Kuwabara, 1967). In the investigations of the manufacturing process with regard to aroma formation, chemical reactions initiated at withering have been found to be important in the formation of volatile compounds during fermentation (Takeo, 1984). Increase in the degree of wither is accompanied by an increase in linalool, a compound that enhances flavour, and a decrease in *trans*-2-hexenal, which is deleterious to flavour (Fernando and Roberts, 1984). However, the major compounds that induce flavour are formed in reactions taking place during fermentation. These reactions culminate at firing, giving rise to some more volatile compounds and resulting in the loss of certain others. Firing process is essential to convert the flowery, greenish aroma of fermented but unfired tea into the characteristic flavoury, black tea aroma. Sanderson and Graham (1973) explained this phenomenon by suggesting that some reactions require unusually high oxidation potentials such as those generated at the firing. Yamanishi *et al.*, (1968a) reported that appreciable losses in alcohols, carbonyls, and phenolic compounds present in fermented tea leaf occurred during the firing.

Amino acids have drawn attention as possible precursors of black tea aroma (Bokuchava and Popov, 1954). The origin of many aroma compounds have been traced back to amino acids contained in the green tea leaf. Using ^{14}C-labelled amino acids in a model tea fermentation system, Co and Sanderson (1970) showed that the α-amino acids present in tea leaf undergo Strecker degradation to form corresponding aldehydes in the presence of oxidizing tea flavanols. Among the fatty acids, linoleic acid has been found to be oxidatively degraded to *trans*-2-hexenal during fermentation. Compounds other than amino acids, fatty acids and carotenoids have not been adequately investigated in relation to their role as aroma precursors. Tea flavanols, which are the major chemical group in the tea leaf, contribute only indirectly to tea aroma by becoming essential for the oxidation of

the carotenoids. Oxidation of flavanols have been reported to be a precondition for most of the black tea aroma-forming reactions (Sanderson *et al.*, 1971).

1.3. Leaf surface microorganisms and flavour in tea

Flavour, of exceptional quality, in Sri Lankan tea is confined to two brief seasons during the months of January-February and July-August in the Dimbula and Uva tea growing regions respectively. Though effective control over the process of aroma biogenesis is commercially desirable, attempts to induce flavour in tea during other times of the year have been largely unsuccessful. Tea industry sources in Sri Lanka believe that the 'pink yeasts' colonizing tea leaves during the flavour season in the Dimbula tea growing region play an important role in flavour biogenesis. This belief however has not been substantiated by sound scientific rationale. The possibility exists that this yeast, *Rhodotorula glutinis*, secretes chemical compounds that enrich tea flavour. The stage of tea manufacture during which the chemical compounds are secreted is of particular importance since any compounds produced on the green leaf surface are bound to undergo chemical changes during the fermentation and firing stages of tea manufacture.

Reports of fungal biosynthesis of volatile compounds are not uncommon (Kempler, 1983). Laboratory cultures of *Trametes odorata* and *Phellinus* sp. have produced sweet, roselike, and sweet roselike aromas corresponding to the biosynthesis of the monoterpenes linalool, geraniol and nerol respectively (Halim and Collins, 1971). Drawert *et al.* (1983) identified linalool and coumarin as the main compounds responsible for the intensive sweet flowery odour of *Pleurotus euosmus* cultures. Linalool and geraniol have been long considered as the most important components of the tea aroma complex and a close correlation between the contents of linalool and geraniol in tea and the market price achieved has been reported (Fernando and Roberts, 1984). Collins and Halam (1970) recovered lactone-6-pentenyl-α-pyrone from the distillates of potato dextrose agar on which *Trichoderma viride* had been cultured. This compound too has been found in the tea aroma complex (Yamanishi *et al.*, 1968a). The yeast *Sporobolomyces odorus* produces a characteristic fruity odour when grown on standard culture medium. Study of the accumulation of volatile metabolites in 34 strains comprising 10 species of the genus *Ceratocystis* has shown that short-chain alcohols and esters, lower terpenes, terpenoids and 2-phenylethyl acetate were produced (Sprecher and Hanssen, 1983). In addition to the above examples of biosynthesis of flavour compounds by fungi, many chemical conversions executed by fungi have been reported. Gehrig and Knight, (1958) reported the

ability of *Penicillium roqueforti* (the fungal species used in blue-cheese making) to convert fatty acids to 2-ketones. The spores of other *Penicillium* spp. and *Aspergillus* spp. have been reported to rapidly convert caprylate to 2-heptanone (Gehrig and Knight, 1961). *Botrytis cinerea*, endemic in some European vineyards, significantly improves the flavour of wines made from the infected grapes. The infection of ripe grapes with *B. cinerea* ('noble rot') is essential for the production of some high quality sweet wines of France. This effect is brought about by the unidentified monoterpenes to which linalool is transformed by *B. cinerea* (Shimizu *et al.*, 1982).

All fungi referred to above have been found on leaf surfaces. However, the *in vitro* biosynthesis reported has taken place under conditions which are not often encountered on leaf surfaces. In the light of discussion in section 1.1.2. significant growth and establishment of these fungi (which are relatively uncommon on leaf surfaces) is unlikely to take place in the leaf surface environment. Chemical substances secreted on leaf surfaces are destined to be converted to different compounds during tea manufacture. The compounds found in the tea aroma complex are conversion products and none of these compounds have been reported in the green tea leaf. A significant contribution by these compounds formed before the manufacturing process cannot be assumed due to changes they are bound to undergo during the manufacturing process. Phylloplane microorganisms could not be assigned a role in tea aroma formation through the direct biosynthesis of tea aroma compounds.

Rhodotorula glutinis, the 'pink yeast' suspected as a potential contributor to tea flavour (by growing on tea leaf surfaces), is a universally occurring phylloplane colonizer. The supposed role of *R. glutinis* on aroma formation has so far not been substantiated by sound scientific rationale or a biochemical pathway. Nakamichi *et al.* (1983) reported that this yeast secretes the enzyme phenylalanine ammonia lyase (PAL). This enzyme is vital for the biosynthesis of flavanols due to its role in the conversion of the amino acid phenylalanine to cinnamic acid. It has also been reported that *R. glutinis* secretes an acid phosphatase enzyme (Watorek and Kwiatkowska, 1983). Participation of this enzyme in the conversion of farnesyl phyrophosphate to nerolidol has been documented (Nishino *et al.*, 1982). Cinnamaldehyde and nerolidol have been identified in the tea aroma complex. Due to its extraordinary success in the leaf surface environment, secretion of such chemical substances on leaf surfaces by the genus *Rhodotorula* is far more plausible than by any of the fungi referred to in the section 1.3. However, the effects of the manufacturing process is certain to undermine potential contributions of these compounds to the aroma complex.

This invalidates the possibility of a contribution (by the yeast) to tea aroma via chemical substances synthesized on the leaf surface, and necessitates novel explanations.

1.3.1. *Rhodotorula glutinis* and tea flavour - A novel hypothesis

Tea leaf carotenoid studies (Tirimanne and Wickremasinghe, 1965; Hazarika and Mahanta, 1983) have indicated an appreciable decrease in the concentration during manufacture. Sanderson *et al.* (1971) showed that the carotenoids content of the green tea leaf (0.053% of the dry weight) decreased during tea manufacture to 0.026% in the fired black tea. In a model system designed to follow the fate of β-carotene during tea fermentation, it was found that β-carotene is oxidatively degraded to β-ionone and several other compounds (Sanderson *et al.*, 1971). Terpene alcohols such as linalool which contribute significantly to tea flavour are also believed to be the products of oxidized carotenoids (Hazarika and Mahanta, 1983). Sweet floral aroma sometimes associated with tea is attributed to these oxidation products of carotenoids. Further, the association of higher quality (flavour) with bright sunny weather is explained on the basis of increased carotenoids biosynthesis under such conditions (Hazarika and Mahanta, 1983). Experiments using ^{14}C-β-carotene have showed that several unidentified compounds also were formed. These results indicate that the carotenoids present in the green tea leaf undergo oxidative degradation during tea manufacture to form important black tea aroma constituents. The yeasts of the genus *Rhodotorula* derive their characteristic pink colour from the pigments β-carotene, γ-carotene, torulene and torularhodin. Quantitative and qualitative analyses of these pigments have been reported by Peterson *et al.* (1954), Peterson *et al.* (1958) and Vaskivnyuk (1985). It could be deduced from the above that the supposed enrichment of tea aroma by *R. glutinis* takes place via β-carotene (and possibly the other carotenoids) contained in the cells. A significant effect could be expected in view of the relatively high contents of β-carotene in the yeast cells. However, the proof of such a hypothesis demands proof of underlying assumptions that the yeast occurs in significant quantities on tea leaf surfaces and biosynthesizes significant quantities of β-carotene on tea leaf surfaces. Biosynthesis of carotenoids under conditions similar to those occurring during the tea flavour season in Sri Lanka (characterised by dry, bright and sunny weather conditions, moderately high temperatures and comparatively large differences between the day and night temperatures) needs to be investigated. This dissertation based on the above hypothesis reports results of investigations into the effect of comparable environmental conditions on the biosynthesis of β-carotene by *R. glutinis*.

Chapters 2 and 3 report the results of studies of the fundamental aspects of phylloplane microflora of *Photinia glabra* and *Camellia sinensis* respectively. In these studies, particular attention has been paid to the critical role played by the methods of study. Chapter 4 focuses on the effects of low water potentials on spore germination and mycelial growth of common phylloplane fungi. Availability of water is considered the factor which limits most, the establishment of fungi on leaf surfaces. The study examined the ability of the most abundant fungi on leaf surfaces to withstand low water potentials which characterize this environment. The central argument presented in this thesis rests on the ability of *R. glutinis* to colonize successfully the leaf surface and biosynthesize β-carotene. Chapter 5 reports an environmental analysis of growth and carotenoids biosynthesis by *R. glutinis*. These results suggest that changes in environmental factors cause qualitative and quantitative changes in carotenoids biosynthesis. This is encouraging in view of the anticipated industrial exploitation of this yeast. The pigment complex of the yeast *R. glutinis* comprises four major carotenoid compounds; β-carotene, γ-carotene, torulene and torularhodin. Conventional methods of separation of these carotenoids (paper chromatography and thin layer chromatography) are fraught with deficiencies that give rise to inaccurate information. High pressure liquid chromatography is being increasingly used in the separation of sugars, proteins and lipids. An introduction to this method and a comprehensive survey of literature are presented in chapter 6. Chapter 7 presents the results of analysis of yeast carotenoids using high pressure liquid chromatography. In this experiment, the variation in the biosynthesis of β-carotene component of the pigment complex was studied. Similar to the total pigment content (chapter 5), the β-carotene content varied in response to changes in environment. This observation indicates that industrial application of *R. glutinis* is a feasible proposition. Carotenoids of the tea leaf (grown in the glass house) were separated using high pressure liquid chromatography. This is the first reported instance of the application of hplc for the analysis of tea leaf carotenoids. In view of the assumed role the carotenoids play in tea aroma formation, this application will be useful in the rapid analysis of tea leaf carotenoids for the comparison of clones, and methods of manufacture. Such comparisons were made between the young and mature leaves and leaves subjected to different intensities of light. The findings suggest that manipulation of *R. glutinis* provides the first opportunity of achieving control over the phenomenon of tea flavour.

Chapter 2

Seasonal variation of phylloplane fungi of *Photinia glabra*

2.1. Introduction

The leaf surfaces of higher plants provide sanctuary to numerous airborne fungal propagules. Experimental evidence suggests that only a fraction of more than thirty fungal taxa reported on leaf surfaces actually colonize this environment. Ruscoe (1971) estimated that fungi occupied less than one per cent of the surfaces of *Nothofagus* leaves. Area occupied by fungi on the leaf surfaces of *Hordeum vulgare* reached 2% in exceptionally favourable conditions (Diem, 1974). Variabilities in the levels of nutrients, temperature, and available moisture, and the presence of inhibitory chemical substances restrict fungal growth on leaf surfaces (Burrage, 1971; Tuckey, 1971; Irvine *et al.*, 1978; Rodger and Blakeman, 1984). Contrary to this situation, the estimates of leaf surface fungal populations obtained by the use of cultural methods of study point to the leaf surface as a site of heightened fungal activity. However, most of these fungal colonies originate from the fungal propagules present inactively on leaf surfaces, and commencing growth in the salubrious environment of the culture medium. Methods of study that distinguish between casual itinerants and the authentic inhabitants of the leaf surface are lacking. Underlying deficiencies of the methods presently employed in phylloplane studies have been discussed by Dickinson (1971) and Lindsay (1976). Direct observation techniques are invaluable in verifying actual fungal growth on leaf surfaces. Use of cultural techniques and supplementary direct observation techniques should yield a composite picture of the phylloplane microflora and its activity.

Phylloplane microflora of various plant species divulge some common characteristics. Studies conducted in the northern and southern hemispheres manifest a phylloplane microflora that is universally similar. A group of 'field fungi' (Hudson, 1968) occur on the phylloplanes of many plant species that have been examined. Most of these numerically important fungi *(Aspergillus, Cladosporium, Penicillium, Trichoderma, Epicoccum*) have been reported on the leaf surfaces of beech (Hogg and Hudson, 1966); Pisum (Dickinson,

1967); larch (McBride and Hayes, 1977); poplar (Andrews and Kenerley, 1980) and many other plants. However, there are few reports of host specific phylloplane fungi. *Ascochytula obiones* was restricted to *Halimione portulacoides* (Dickinson, 1965) and *Hyalodendron* sp. to *Quercus robur* (Tsuneda and Skoropad, 1978).

Conflicting reports of differences between population numbers and composition of the microflora on upper and lower surfaces of leaves are available in the literature. A higher degree of mycelial growth of *Cladosporium* on the lower surface was observed by McBride and Hayes (1977) and Andrews and Kennerley (1980). Rodger and Blakeman (1984) reported that numbers of all fungi other than *Cladosporium* on the upper and lower surfaces of sycamore leaves were equal. Fumagoid fungal colonies were more numerous on the upper surfaces of the leaves of *Nothofagus truncata* (Ruscoe, 1971). In contrast to these reports, equal population levels of yeasts and fungi on the upper and lower surfaces were recorded by Last (1955), Apinis *et al.* (1972), Mishra and Dickinson (1981) and Hayes (1982). Populations of yeasts on the two surfaces of apple leaves have been reported to be equal (Andrews and Kenerley, 1980).

Important qualitative and quantitative effects of the seasons on phylloplane microflora have been observed in Pisum (Dickinson, 1967), *Hippophae rhamnoides* (Lindsey and Pugh, 1976), and sycamore (Rodger and Blakeman, 1984). These studies report a remarkable increase of yeasts and a less marked increase of mycelial fungi during summer. The microflora on the leaves of larch (McBride and Hayes, 1977) and rapeseed (Tsuneda and Skoropad, 1978) have not exhibited such seasonal variations.

Lack of confirmity among studies of the phylloplane microbiology suggest that the plant species and the location partially determine the composition, topographic distribution and seasonal variation of the microflora. A study of the active microflora colonizing leaf surfaces of a comparable plant species, and the factors governing population variations of these fungi, will be relevant to the central argument put forward in this thesis regarding the role of phylloplane yeasts in tea aroma biogenesis. With the use of a combination of direct and cultural techniques, the microflora colonizing green leaves of *Photinia glabra* (growing in Canberra), and the factors governing variations in populations were studied.

2.2. Materials and Methods

2.2.1. Leaves

The leaves were collected fortnightly for a period extending from May to September 1985 from three *Photinia glabra* plants grown at Weston Park, Yarrulumla, A.C.T. *Photinia glabra* is an evergreen ornamental plant which assumes a bushy growth habit and grows to a height of 2-4 m. A preliminary evaluation of the microflora on the first, second and third leaves from the shoot apex indicated that the microflora on the three leaves did not differ significantly. Therefore the first leaf was sampled. Standardization of sampling was such that similar sample points were used on all three trees throughout the experiment. The sample comprised 45 leaves. Leaves were severed from the shoots with a sterile scalpel into sterile polythene bags, and brought to laboratory where the microflora was isolated in less than 24 h.

2.2.2. Evaluation of culture media

Due to the significance of culture medium in the isolation of leaf surface microflora, a preliminary evaluation of the suitability of four commonly used culture media, Potato Dextrose Agar (PDA), Czapek-Dox Agar (CDA), Corn Meal Agar (CMA) and Oat Meal Agar (OMA), was undertaken. Twenty replicate plates of each medium (prepared according to the standard procedures) were inoculated with the leaf print technique. After incubating for 5 d at 25°C the total number of microbial colonies was recorded and the fungi and the yeasts taxonomically classified into major groups. Potato Dextrose Agar was chosen as the medium which facilitated the growth of the widest range of fungi and yeasts.

2.2.3. Methods of isolation of microorganisms

Importance of employing a combination of cultural and direct observation methods to isolate phylloplane fungal propagules was demonstrated by Wickremasinghe *et al.* (1985). With a view to examining the efficacy of various cultural techniques in removing fungal propagules from the leaf surfaces of *Photinia glabra*, the techniques were compared in a preliminary experiment. The surface microflora of 20 leaves was isolated on to PDA plates by the leaf washing technique. Impressions of the washed leaves were made on solidified agar. The leaf print technique was used to isolate the microflora on a group of 20 leaves. (Details of these methods of isolation are given below). Based upon the results of this study it was decided to employ leaf washing and print methods.

Leaf washing: In the washing method, 10 whole leaves were shaken in 100 ml of sterile water (in a 250 ml conical flask) in a wrist action shaker for 30 min. The degree of shaking was arbitrarily determined to obtain maximum turbulence without spillage and this degree of shaking was used for all isolations by this method. Samples of 10 leaves provided a fungal population that was representative and convenient to handle. Aliquots (0.1 ml) of washings were spread on the solidified agar surface with a sterile inoculating loop. Twenty replicate inoculations of the washings were done. Leaf area (upper and lower) was measured before the leaves were discarded. Colony development was observed after 5 d of incubation at 25°C and the number of colonies of each fungal species recorded. Fungal colonies were isolated on to PDA slants for identification. The number of colonies were expressed per unit area of leaf surface.

Washed leaf prints: Impressions of the leaves used in the leaf washing method were made on agar plates to examine the extent of removal of propagules by the leaf washing method.

Leaf prints: This method, originally used by Potter (1910), was the most reproducible of all techniques employed in this study. Ten leaves were bisected along the mid rib and the halves were firmly pressed on to the surfaces of solidified culture medium (PDA) to leave an impression of the leaf surface. Special care was taken to expel all air bubbles between the surfaces, since this was found essential for the isolation of all microorganisms. Two halves of each leaf were used to sample the adaxial and abaxial surface microflora. Petri plates were incubated at 25°C for 5 d and the colonies were counted and isolated for identification. Leaf area was measured and the results expressed as number of colonies per unit leaf area.

Sporefall technique: This selective technique, originally used by Last (1955) was used to isolate the yeasts which forcibly discharge their spores. Ten leaves were bisected along the midrib and pasted on to the lid of the Petri dish with one half exposing the upper and the other the lower surface to the medium. After incubation for 48 h the leaves were removed and the plates incubated for a further 3 d before examination.

Direct light microscopy: Surfaces of 10 leaves were thinly coated with nail polish (by dipping) and allowed to dry for 1 h under lamina flow. Each leaf was then cut into 1 cm^2 segments and the nail polish strip was peeled. Ten randomly selected fields from each of 10 randomly selected squares were observed under the light microscope (x 100), after

staining with cotton blue - lactophenol. Results were expressed as the mean number of propagules per unit area of leaf surface.

Scanning Electron Microscopy: Ten leaves were cut in to 1 cm^2 squares avoiding the mid rib. These leaf squares were immersed in liquid N_2 for 20 min and placed overnight in a Dynavac freeze drier under vacuum at -40°C. The leaf squares were placed on SEM specimen stubs, sprayed with a 200 A layer of gold. Upon drying they were observed at 30 kV in a Cambridgescan S180 Scanning Electron Microscope. Preparations were made with either adaxial or abaxial surface of leaves uppermost.

Meteorological data: Daily charts of maximum and minimum temperature, relative humidity and rain fall data were obtained from the A.C.T. Regional Office of the Weather Bureau. Weather data had been recorded at a site approximately 1 km from the site of sampling. Climatic variables are presented as means for the fortnight preceding sampling date, except for rainfall which is expressed as fortnightly total. Correlation analysis was carried out by the Genstat statistical package.

Identification of fungi: Majority of fungi isolated were common Fungi Imperfecti and were identified in the laboratory. Specimens of rare fungi were sent to the Commonwealth Mycological Institute, Surrey, England for identification. The yeasts were identified by the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

2.3. Results

Evaluation of culture media and Methods of isolation: Total number of microbial colonies and the number of colonies of each fungal group occurring on the four culture media are given in Table 2-1. In respect of the total number and the variety of fungi sustained, PDA appears far superior to the other media tested. PDA facilitated the growth of highest number of colonies of the predominant fungal group on leaf surfaces, the Fungi Imperfecti. Among this group of fungi however, *Epicoccum nigrum, Alternaria alternata* and *Cladosporium cladosporioides* constituted nearly 95% of the population. Many of the other fungi belonging to this group occurred infrequently. Relatively low number of colonies of fungi belonging to Ascomycetes or to Basidiomycetes reflect the scarcity of these fungal groups on the phylloplane rather than the inability of the media to sustain them. The only ascomycetous fungus encountered in this study was *Chaetomium globosum* which was recorded in small numbers on numerous sampling dates. The yeast *Sporobolomyces roseus* was the predominant member of the Basidiomycotina. (Spores of

Medium	Phyc	Asco	Basi	F.Imp	Total
PDA	0	1	2	14	17
CMA	0	0	1	4	05
OMA	2	0	1	3	06
CDA	2	1	0	6	09

Phyc = Class Phycomycetes, Asco = Class Ascomycetes, Basi = Class Basidiomycetes, F. Imp = Fungi Imperfecti, PDA = Potato dextrose agar, CMA = Corn meal agar, OMA = Oat meal agar, CDA = Czapeck-Dox agar

Table 2-1: Number of fungal colonies isolated by different culture media

Agaricus were observed in the direct microscopic study). Based on these results PDA was used as the culture medium for periodic samples.

The estimates of the three major fungal species inhabiting the phylloplane of *Photinia glabra* at this location (*Epicoccum nigrum, Alternaria alternata* and the yeasts) provided by the leaf washing, washed leaf prints and leaf print techniques are given in Figure 2-1. While leaf prints isolated the highest proportion of the populations of all fungi and yeasts, leaf washing method provided the lowest estimates. In the case of yeasts, the relative efficiencies of the three techniques appear to differ least, though with significantly lower population estimates provided by the washing method. Estimates provided by the washed leaf prints almost amount to the differences between the washing and impression techniques. All three techniques reveal seasonal trends of the microflora without distortion. The significant increase of *E. nigrum* and the populations of the yeasts from the 4 May to 11 May and the reductions in populations between 25 May and 30 May have been conveyed by all methods. However, on a number of sampling dates contrasting population estimates have been provided by the different techniques.

Sample characteristics: Relatively high variation of fungal counts among replicate leaves was observed on all sampling dates. Table 2-2 exhibits this variation within samples. Large values of standard deviation and standard errors could not be reduced by increasing sample size in the manageable range. Variation was greater in yeast populations than in mycelial fungi. The variation in total microbial population has been largely due to the variation in yeast populations. Consistently high *E. nigrum* populations have exhibited comparatively little variation.

2.3.1. Fungal and yeast populations on the leaf surfaces of *Photinia glabra*

The fungi observed were those commonly associated with the phylloplanes of other plants universally. Leaf surfaces of *Photinia glabra* at Weston Park, Yarrulumla, A.C.T., were colonized predominantly by three major species of fungi; *Epicoccum nigrum, Cladosporium cladosporioides* and *Alternaria alternata*. Populations of these fungi dominated the leaf surfaces throughout the sampling period. Among the yeasts, *Sporobolomyces roseus, Rhodotorula glutinis* and *Cryptococcus albidus* were predominant. *Aureobasidium pullulans*, a yeast frequently reported from the leaf surfaces of many plants, was not encountered. Table 2-3 enumerates the fungi recorded by cultural techniques from the upper and lower surfaces.

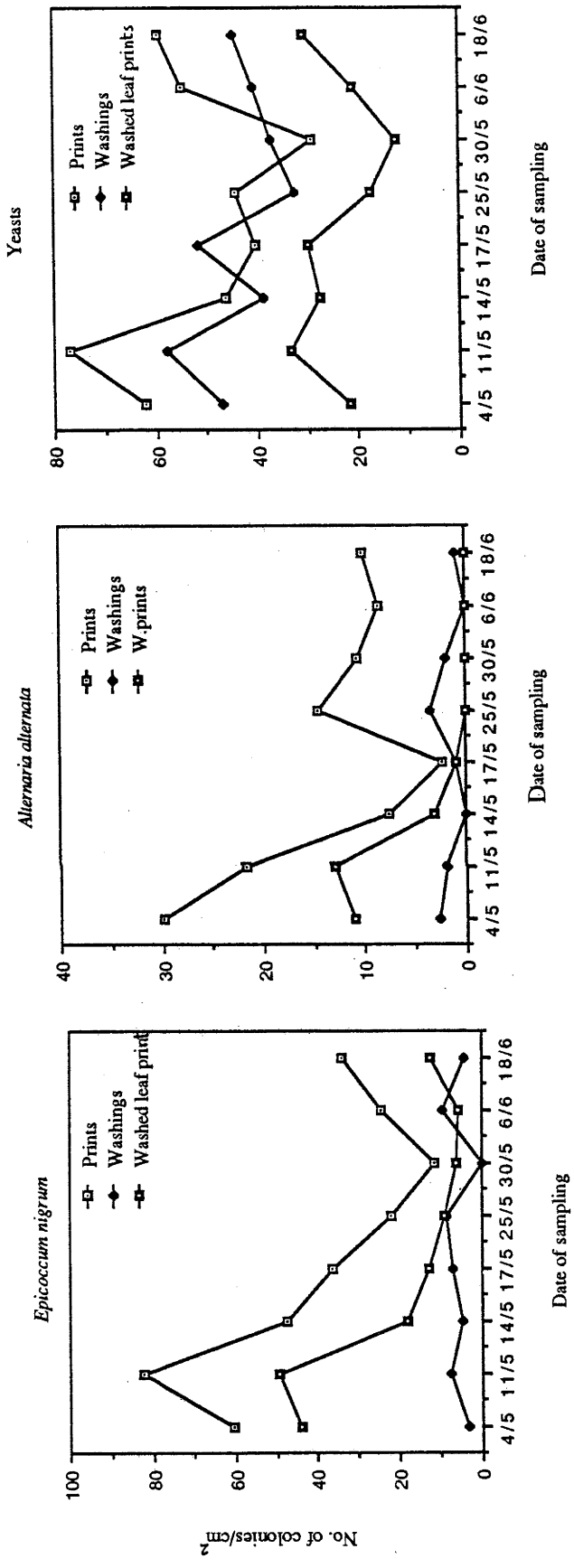


Figure 2-1: Estimates of fungal populations obtained by different methods of isolation

	$\bar{Y}$	S. E.	$S_{\bar{y}}$	95% C. I.
Epicoccum nigrum	28.4	8.54	3.82	28.4 ± 10.6
Alternaria alternata	11.2	6.45	2.88	11.2 ± 7.99
Yeasts	67.2	36.8	16.4	67.2 ± 20.4
Total microorganisms	106.8	35.47	15.8	106.8 ± 44.0

$\bar{Y}$ = Mean number of colonies, S.E. = Standard error, $S_{\bar{y}}$ = Standard deviation of a mean, C. I. = Confidence Interval

Table 2-2: Sample statistics of phylloplane microbial populations

	Number of colonies/cm^2															
	1/5		15/5		30/5		15/6		30/6		15/7		30/7		15/8	
	U	L	U	L	U	L	U		U	L	U	L	U	L	U	L
Epicoccum nigrum	61	05	47	09	26	11	39	12	32	06	45	08	106	09	77	10
Alternaria alternata	03	12	04	09	06	21	04	24	39	32	21	27	17	28	19	23
Botrytis cinerea	-	03	-	-	01	01	03	-	-	-	27	14	22	09	14	13
Nigrospora sphaerica	07	-	02	-	01	-	01	-	04	02	03	-	05	-	04	-
Phoma sp.	01	-	02	-	-	-	-	-	02	-	02	-	01	-	-	-
Microspora indicum	-	01	01	-	-	-	-	-	01	-	01	-	-	01	-	-
Mucor haemalis	02	04	02	01	-	-	01	-	03	01	03	-	02	01	01	-
Chaetomium globosum	-	-	01	-	-	-	-	-	-	-	01	-	-	-	-	-
Fusarium solani	01	01	08	01	-	-	-	-	06	01	-	-	-	-	04	02
Rhodotorula glutinis	42	46	13	19	17	24	11	17	19	26	20	22	16	19	14	24
Cryptococcus laurentii	06	09	11	10	03	05	01	01	02	03	01	02	01	01	01	04
Sporobolomyces roseus	19	15	08	09	14	06	08	10	04	03	10	05	06	02	08	04

Table 2-3: List of fungi and their population numbers isolated from the leaf surfaces of *Photinia glabra*. U = Upper, and L= lower leaf surface

On many sampling dates *E. nigrum, C. cladosporioides* and *A. alternata* collectively accounted for approximately 90% of the population. Although some 20 species of filamentous fungi were isolated, few occurred consistently from one sampling date to the next. Among the yeasts, *S. roseus* and *R. glutinis* outnumbered other species. The number of yeasts was limited to these two species and two *Cryptococcus* spp. Sporefall method isolated *S. roseus* in pure culture due to the exclusive nature of this method. The proportion of germinated spores (as revealed by direct observations) on the upper surface did not exceed 5% of the total number at any stage during the sampling period. Scanty microbial growth on the upper surface was revealed by the same technique.

2.3.2. Microflora on the upper and lower surfaces

The population levels of the major fungal inhabitants of the upper and lower leaf surfaces differed greatly. Table 2-4 presents the results of an analysis of variance of colony counts of two predominant fungal spp. and yeasts on the upper and lower leaf surfaces. Differences (between the upper and lower surface populations) of *E. nigrum* and *C. cladosporioides* were highly significant ($P < .05$) whereas the difference between the two yeast populations was nonsignificant. In all cases the estimates of upper leaf surface populations were higher than those of the lower surface. These data provided by the cultural techniques, however, was not an indication of higher fungal activity on the upper surface of the leaf. Observations made with the direct methods provided contradictory evidence.

Scanning Electron Microscopy: Figs. 2.2-2.4 illustrate a higher degree of fungal activity on the lower surface of the leaf. Figure 2-2 shows the extensive mycelial growth occurring in the vicinity of stomata, on the lower leaf surface. A germinating spore of *E. nigrum* is shown in Figure 2-3. Such germinating spores of this fungal species were rarely observed on the upper surface.

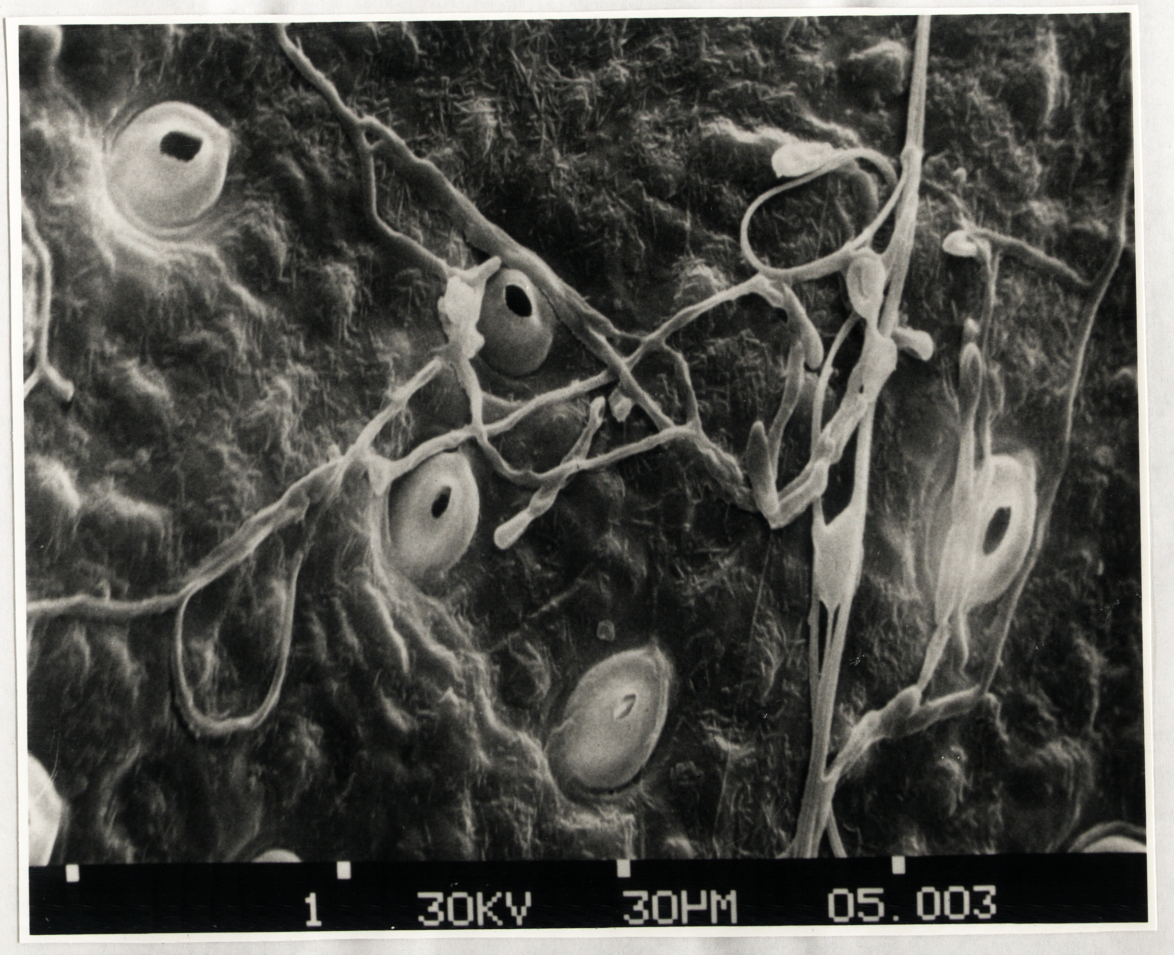
Figure 2-3 shows the mycelium surrounding stomata. Figure 2-4 clearly demonstrates that the yeasts actively grow and reproduce on the under surface of the leaf. Figures 2-5 and 2-7 are the SEM photographs of the upper leaf surface. Relative lack of mycelial growth on this surface was obvious. The maximum mycelial growth was limited to the levels shown in Fig. 2.5 (a) and (b). These photographs and the ones in Figure 2-6 suggest that fungal spore germination take place in the environment of the upper leaf surface. However mycelial growth seems to be restricted by the hostile conditions. Fungal spores

	DF	SED	Mean^{u}	Mean^{l}	F Value
			(no.of colonies/cm^2)		
Epicoccum nigrum	19	19.08	45.9	8.3	23.262**
Cladosporium cladosporioides	16	14.82	19.4	38.5	9.96**
Yeasts	22	25.93	54.4	43.3	1.10^{ns}

DF = Degrees of freedom, SED = Standard error of a diffrerence between means, Means ^{u} and ^{l} are the values for the upper and lower leaf surfaces respectively

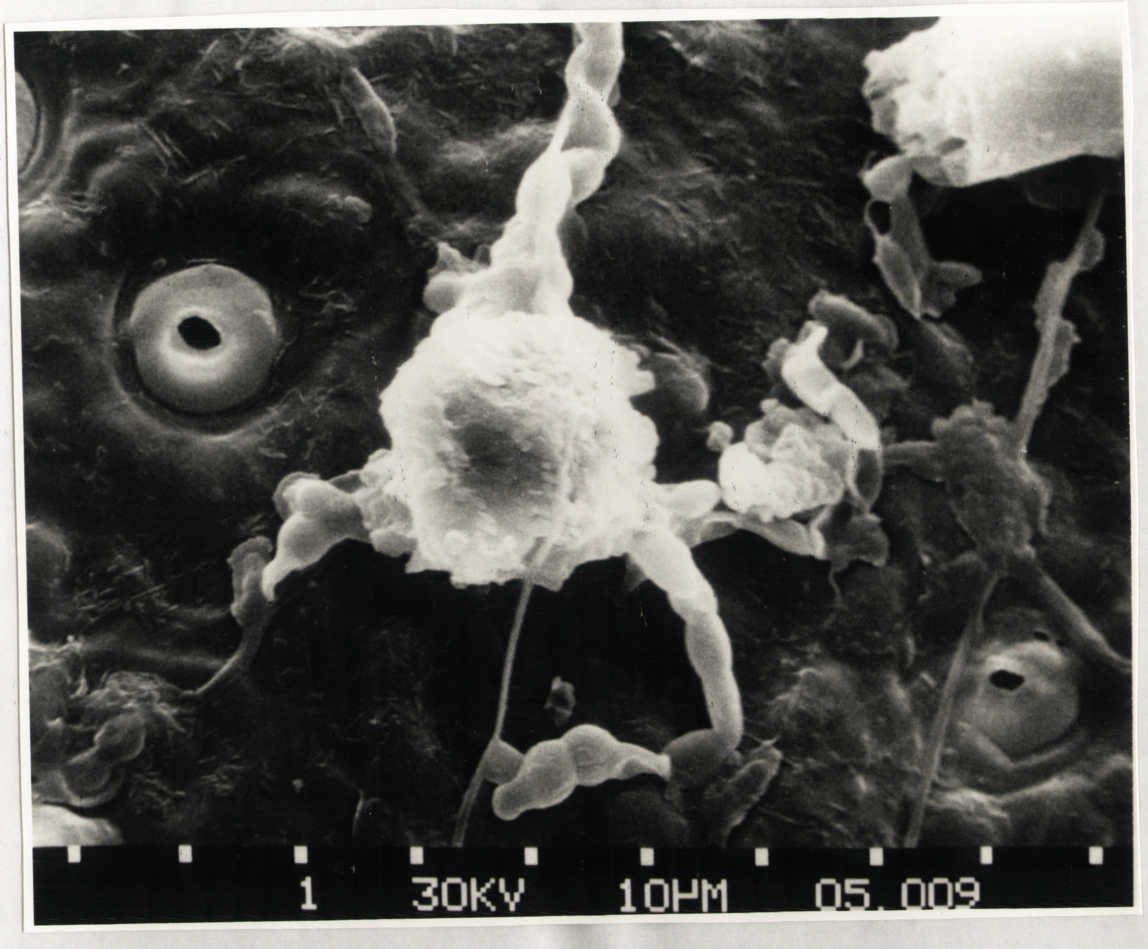
Table 2-4: Analysis of variance of the microbial populations on the upper and lower leaf surfaces of *Photinia glabra*

Figure 2-2: Scanning electron micrographs of the lower leaf surface of *Photinia glabra* exhibiting extensive mycelial growth in the vicinity of stomata


1 30KV 30µM 05.003


1 30KV 10µM 05.010

Figure 2-3: Scanning electron micrographs of the lower leaf surface of *Photinia glabra* a. fungal mycelia around stomata b. a germinating spore of *Epicoccum nigrum*


1 30KV 10μM 05.009

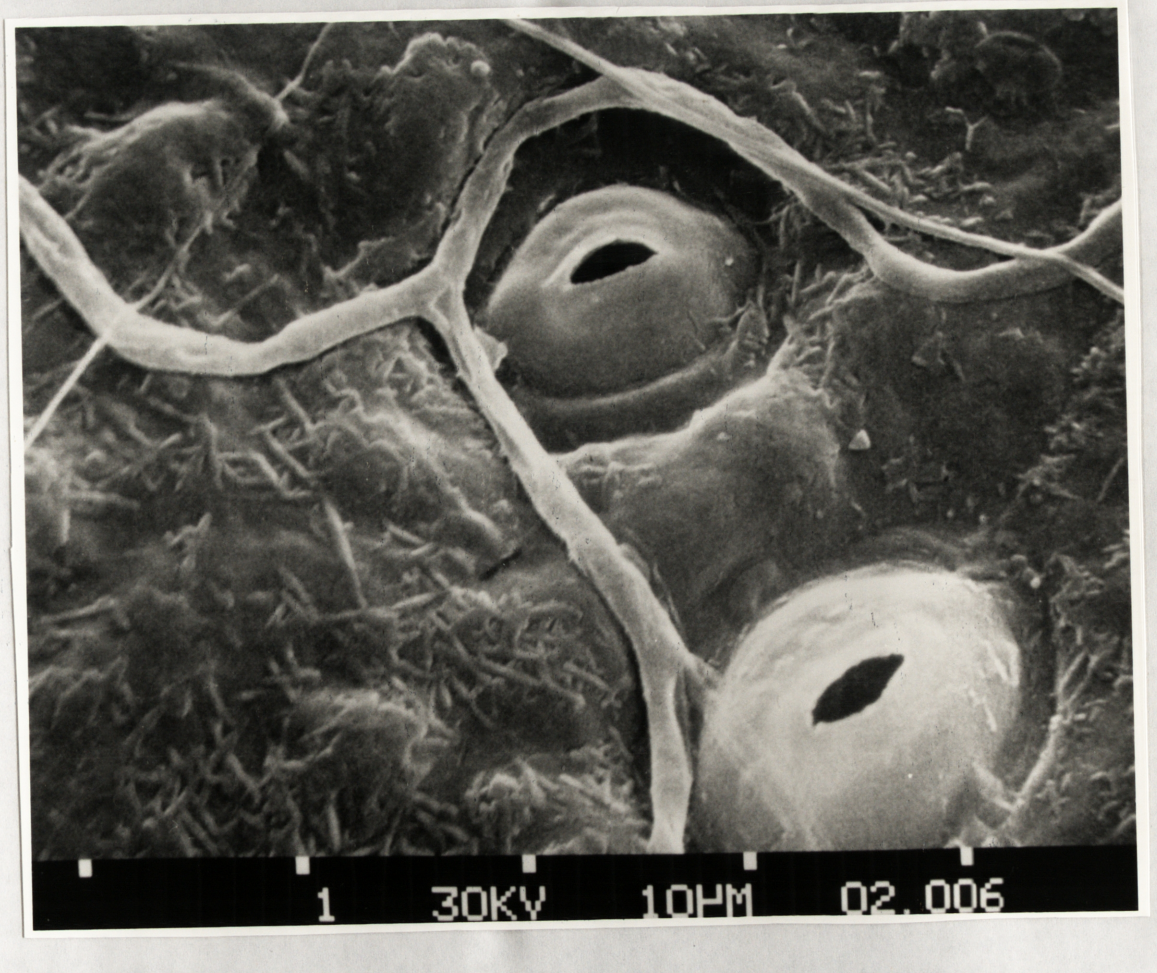

1 30KV 10μM 02.006

Figure 2-4: Scanning electron micrographs of the lower leaf surface of *P.glabra* demonstrating active colonization by yeast and fungi


1 30KV 30PM 05.008

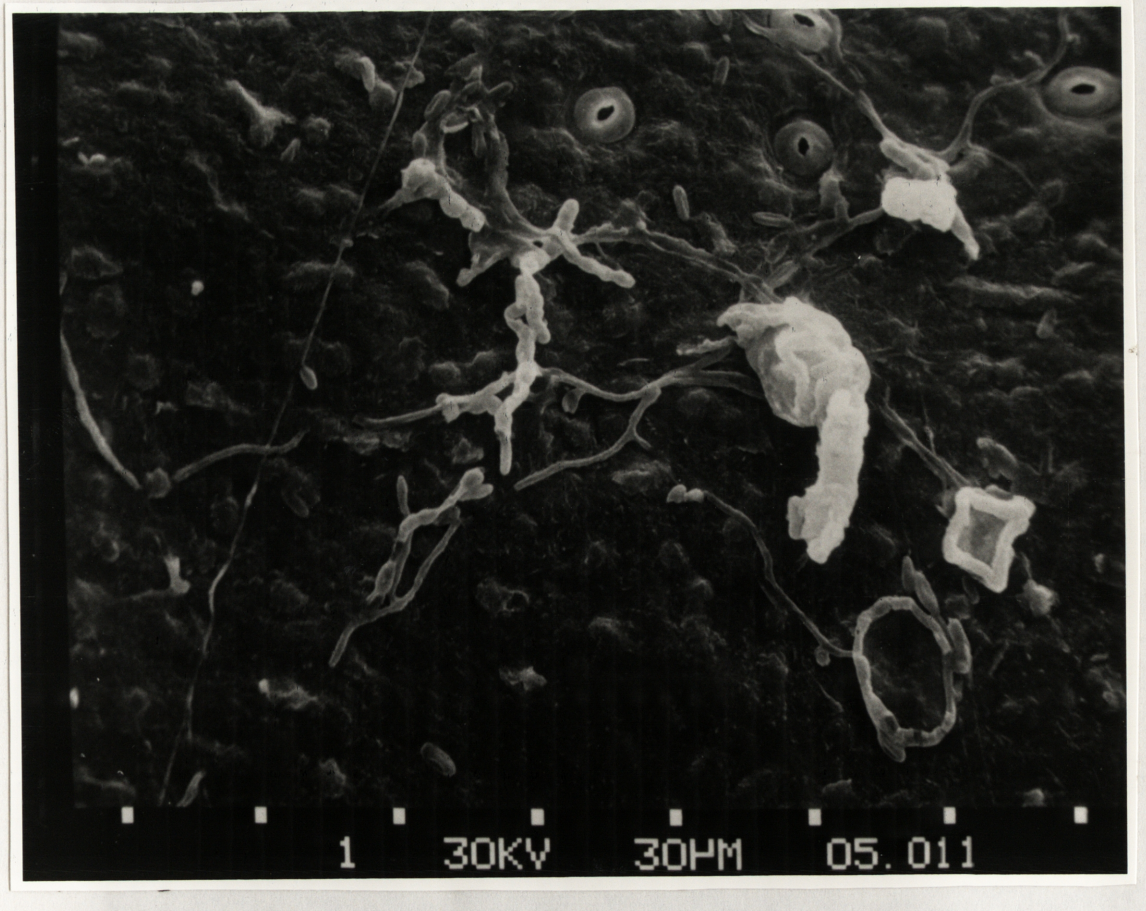
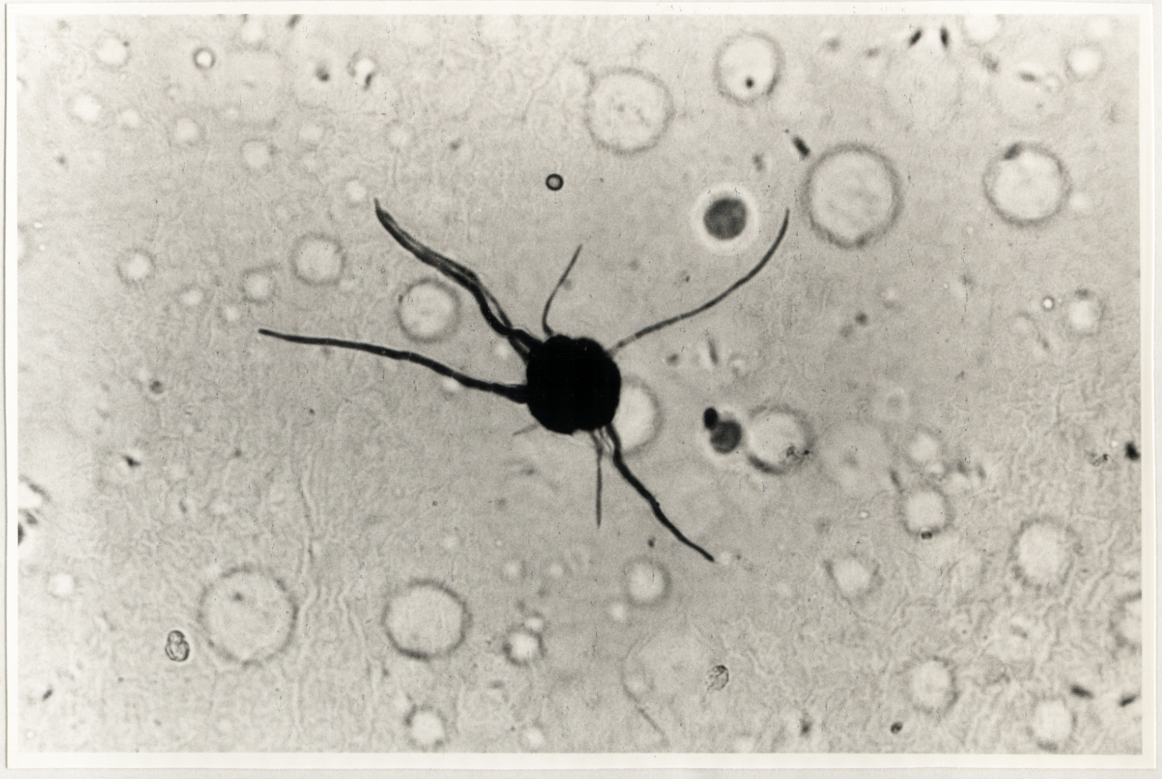

1 30KV 30PM 05.011

Figure 2-5: Scanning electron micrographs of germinating fungal spores on the upper leaf surface of *P.glabra* a. *E. nigrum*, b. *C. cladosporioides*



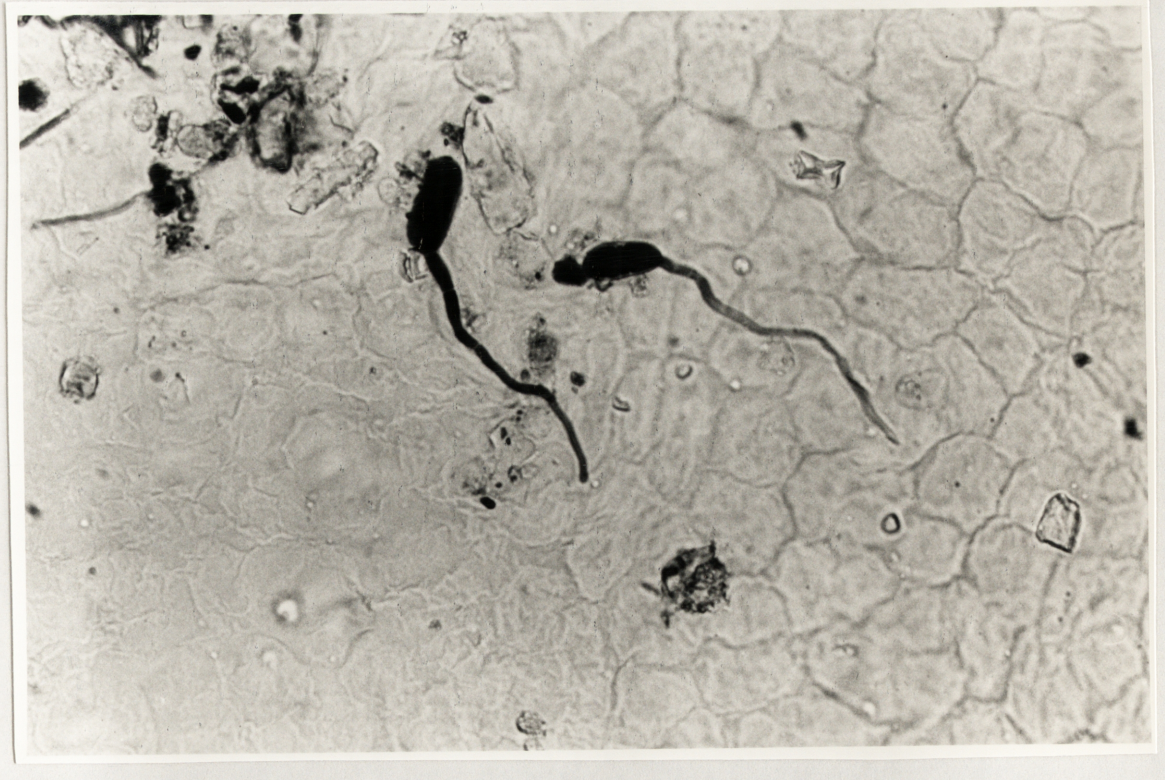
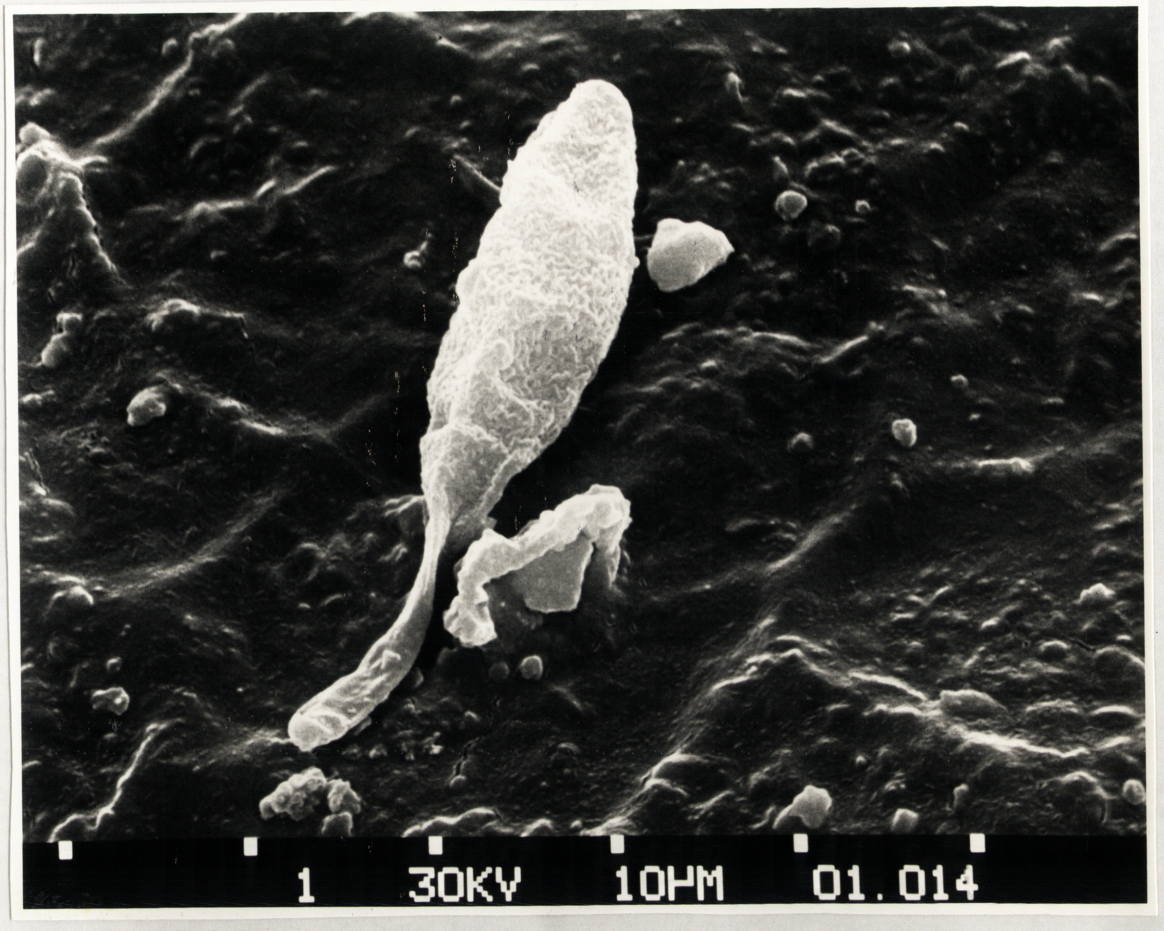


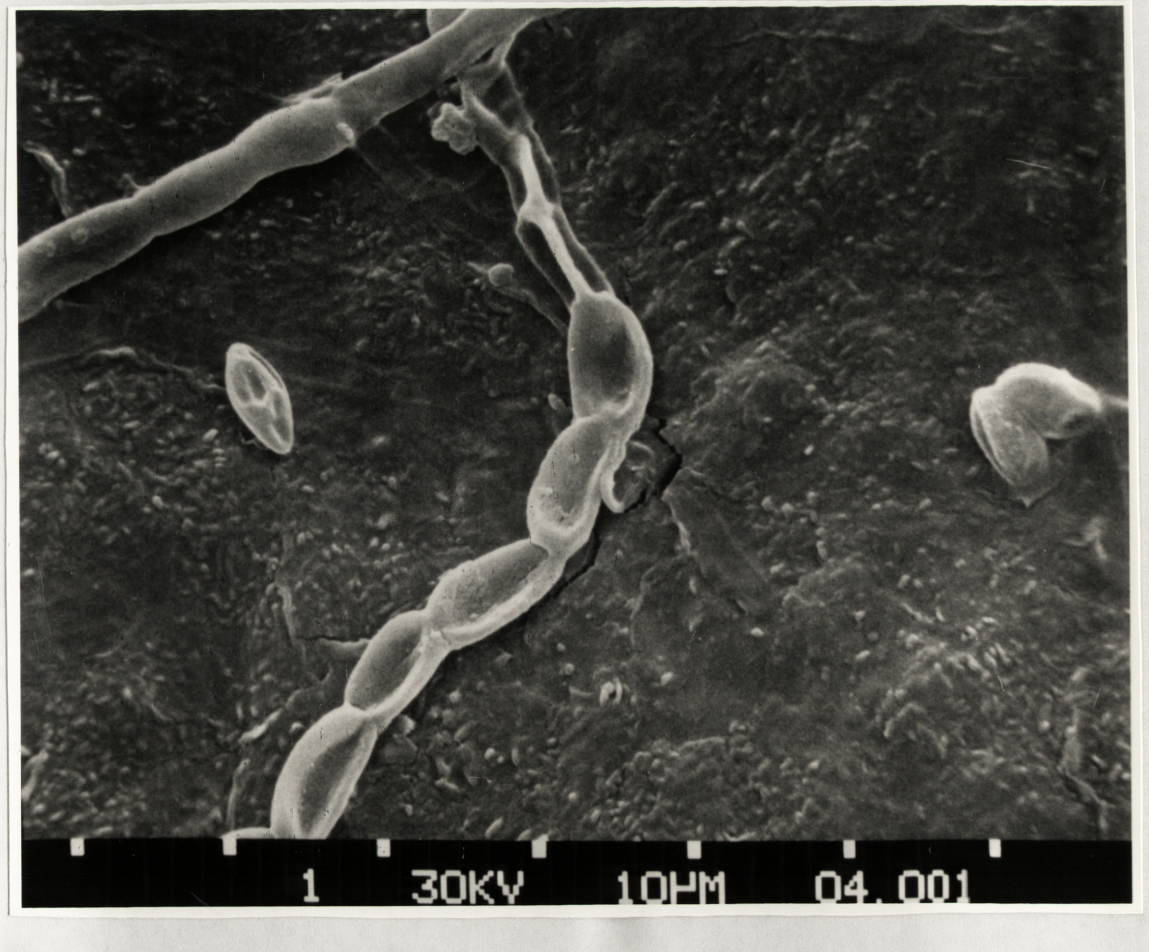
Figure 2-6: Scanning electron micrographs of the upper leaf surface of *P. glabra* a. a germinated fungal spore b. degenerating fungal mycelia


1 30KV 10PM 05.006


1 30KV 10PM 04.009

Figure 2-7: Scanning electron micrographs of the upper leaf surfaces of *P. glabra* a. a degenerating spore of *A. alternata* b. degenerating yeast cells


1 30KV 10UM 01.014


1 30KV 10UM 04.001

in an inactive or degenerating stage are common on the upper surface. A spore of *A. alternata* and yeast cells in a similar state are depicted in Figure 2-7. Many hyphae shrivelled during the SEM sample preparation and observation processes. No sporulating colonies of fungi were observed at any stage. Fungal propagules on the upper surface existed as spores and therefore, were easily isolated by the cultural methods. Yeast populations do not exhibit such differences. Cultural methods, in general, provided misleading information with respect to fungal activity on the phylloplane. The greater number of colonies isolated from the upper leaf surface suggested that fungal activity is higher on this surface than on the lower surface. Direct observations proved otherwise.

2.3.3. Relationship between microbial populations and meteorological data

Figure 2-8 shows the variation of selected weather parameters during sampling period. Variations in rainfall and relative humidity followed a similar pattern, both remaining low from the beginning of May to mid-July, and increasing from then until the end of sampling period. The difference between the maximum and minimum temperatures however, exhibited an inverse relationship to rainfall and relative humidity. Figure 2-9 shows the variations in microbial populations during the sampling period. All populations are positively correlated with the difference between maximum and minimum temperatures. A correlation analysis using Genstat statistical package demonstrated highly significant relationships between the total microbial population, *E. nigrum* population and weather parameters. The populations of *C. cladosporioides* and the yeasts did not exhibit a similar relationship. As shown in Table 2-5 the total microbial population and *E. nigrum* populations have high correlation coefficients with all weather parameters, indicating a strong influence of these factors. *Cladosporium cladosporioides* showed least correlation with rainfall, followed by the difference between maximum and minimum temperatures. The populations of the yeast exhibit higher correlation (yet nonsignificant) with rainfall and relative humidity.

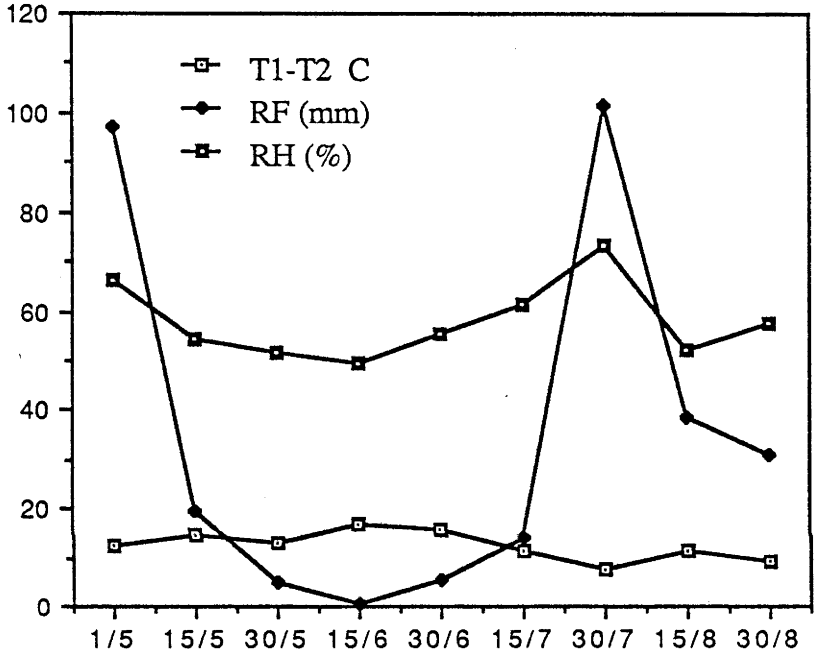


Figure 2-8: Variations in selected meteorological factors during sampling period (T_1-T_2 = Difference between the average daily maximum and minimum temperatures, RF = Total rainfall (mm), RH = Average relative humidity during the fortnight preceding the sampling date

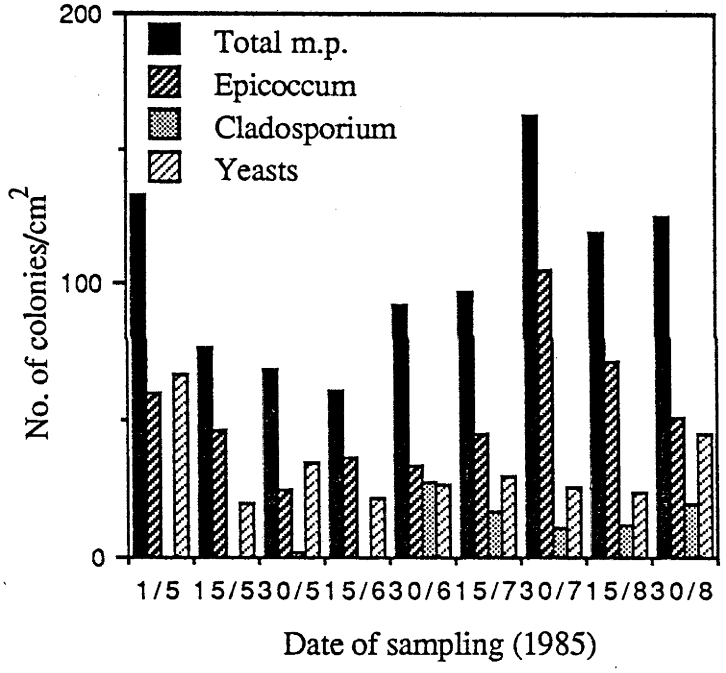


Figure 2-9: Microbial populations on leaf surfaces of *Photinia glabra*

	T_1-T_2	RF	RH
Total microbial pop.	0.8364*	0.8795*	0.8234*
E. nigrum	0.7481*	0.8339*	0.7456
C. cladosporium	0.1642	0.0842	0.2796
Yeasts	0.2366	0.4807	0.3817

T_1 and T_2 = Maximum and minimum temperatures respectively, RF = Total rainfall for the fortnight preceding the date of sampling, RH = Average relative humidity for the fortnight preceding the date of sampling, * = Significant at $P< 0.05$

Table 2-5: Correlation coefficients of selected meteorological factors with phylloplane fungal populations

2.4. Discussion

The composition and temporal variation of the phylloplane microflora of *Photinia glabra* conforms to the general pattern displayed by those of other plant species. Limitations in the cultural methods widely employed in phylloplane studies have been highlighted by the results. The need to employ several cultural techniques in studies of the phylloplane microbiology is demonstrated by the contrasting population estimates provided by these techniques. Advantages of using a range of techniques in quantitative studies of the phylloplane have been elaborated in earlier studies (Lindsey and Pugh, 1976). The leaf print technique appears to provide more credible data, though this method fails to achieve complete isolation of the microflora. Leaf washing technique provided extremely low estimates of the microflora. Incomplete spore removal rather than biased sampling of washings would have caused such low population estimates by this technique. This was confirmed by the residual microflora (which constituted a high proportion of the total microflora) on the washed leaf surfaces and isolated by the leaf print technique. All methods accurately record seasonal trends in fungal population variations, suggesting that any single cultural method could provide reliable data regarding seasonal variations of the phylloplane microflora. However, the suitability of a sole cultural method in studies aimed at more precise quantitative data is questionable. A possible solution in such cases would be to adopt a combination of techniques that complement each other. The leaf washing technique and leaf print technique could be employed in tandem to achieve near complete isolation of the microflora. The summation of the counts provided by the two techniques could be regarded as a more accurate account of the microflora on leaf surfaces.

Scanning Electron Microscope is a useful tool in the verification of actual fungal growth on the leaf surface. It provides information regarding the forms of fungal propagules on leaf surfaces, their spatial distribution and most importantly, their physiological stage. In the present experiment, cultural methods indicated a preponderance of *E. nigrum* on the leaf surface. An interpretation based on this information would have been erroneous. The picture provided by the SEM, of other fungi actively growing on the leaf surface, was crucial in correctly evaluating the leaf surface as a substrate for microbial growth. Samples can be examined under the SEM while the natural distribution of microorganisms on the leaf surface is preserved. This is achieved by obviating the need for bleaching, essential in the light microscopic examination. However, the sample preparation procedure involved in the SEM can cause collapse of fungal propagules. This peril can be avoided by paying due care. The major drawback of the SEM is its inability to facilitate examination of the

whole leaf surface. In the absence of this facility, information gained may not appear interconnected. It is also a relatively expensive method in terms of costs involved in sample preparation. Scanning Electron Microscope emerges as a method that could provide details necessary to explain uncertainties regarding fungal activity on leaf surface. However, this does not warrant its routine application in every study of the phylloplane.

Routine isolation of the microflora on to a commonly used synthetic culture medium could demonstrate only a section of the microflora present on leaf surfaces. Using tap water agar, Dickinson (1973) did not record the presence of *Aureobasidium pullulans* on barley leaves, yet recorded high counts of this yeast when leaf washings were plated on PDA. In addition to such complete exclusions in culture, some organisms favoured by the sources and composition of nutrients in a particular culture medium could overgrow certain others. Differences among the microflora sustained by different culture media in this experiment could be explained on the basis of differences in nutrient composition and carbon sources. CDA and PDA, the media which sustained higher number of colonies are comparable in their contents of major nutrients (Na, K, Mg, Fe) except carbon. The reason for the superiority of PDA over CDA could be the readily available carbon in PDA. Dextrose, a sugar easily absorbed and ingested, could encourage the germination and growth of many of the Fungi Imperfecti. The source of carbon in CDA, sucrose, has to be enzymatically hydrolysed before absorption. Inadequate carbon supplies would have hampered germination and growth of organisms on CDA during the 5 d incubation. Corn Meal Agar and OMA obviously do not contain the range of nutrients needed to support the growth of such a diverse group of fungi. Practical implication of this observation is that sampling of leaf surface microflora has to be done on a number of media in order to choose the medium which renders the broadest spectrum of fungi. Yet, many of the reported studies of the phylloplane have been carried out without adequate attention paid to these aspects. Variation among replicates indicate the dependance of leaf surface microflora on extraneous factors which determine the size and composition of the aerial microflora. The populations of *E. nigrum* and *A. alternata*, which occurred mainly as spores would have reflected their concentration in the aerial microflora. The fungal propagule content in the atmosphere is determined by the environmental and other factors governing growth and sporulation of fungi elsewhere. The variation in yeast populations, which were found to be actively growing on leaf surfaces, could be attributed to different degrees of isolation achieved in each culture plate and the variation in the proportion of yeasts in reproductive stage. Yeasts are generally the most difficult group of microorganisms to isolate by the leaf

print method. Substantial numbers of yeast cells have been found to remain on leaf surfaces after ten repeated prints were made on agar (Parberry *et al.*, 1981). Such inconsistencies would have contributed to the high variation in data. The yeasts in reproductive stage are comparatively easily isolated due to the easy detachment of the buds. However, the proportion of yeast cells in reproductive stage may be subject to unstable conditions in the boundary layer.

Species composition of the fungi on leaf surfaces of *Photinia glabra* is similar to those found on other plant species. The genera frequently isolated from leaf surfaces form the bulk of the air spora (Last, 1955; Collins and Hayes, 1976). The fungal flora is dominated by the three species *E. nigrum, C. cladosporioides* and *A. alternata.* Among the yeasts, *R. glutinis, S. roseus* and *C. albidus* predominated. The occurrence of fungi mainly in the form of spores (with the exception of *C. cladosporioides*), suggests that fungal growth on leaf surfaces is restricted. A feature of the microflora recorded on these leaf surfaces is the absence of *Aureobasidium pullulans.* This yeast is generally regarded as the initial colonizer of green leaves and has been reported on most leaf surfaces that have been examined. However, *Aureobasidium pullulans* has been absent on few plant species including *Acer platanoides* (Breeze and Dix, 1981) and *Ilex aquifolium* (Mishra and Dickinson, 1981). In a comparative study of the phylloplane microflora of *Paspalum dilatatum, Salix babilonica* and *Eucalyptus stellulata, A. pullulans* was not recorded on any of the three leaf surfaces (Lamb and Brown, 1970). Significantly, in all cases where *A. pullulans* has not been recorded, *E. nigrum* has been the major inhabitant of the leaf surface.

Significant differences in the patterns of colonization between the upper and lower surfaces have also been reported by Ruscoe (1971), Wildman and Parkinson (1979) and Mishra and Dickinson (1981). In these studies, though higher numbers of fungal propagules were consistently recorded on the upper surface, a higher degree of spore germination and hyphal growth was observed on the lower surface. Differences between the microenvironments of the upper and lower surfaces explain this phenomenon. Lower leaf surfaces are known to maintain higher levels of relative humidity due to stomatal transpiration. Drying effect of the solar radiation on lower leaf surfaces is reported to be slower due to rougher microtopography. This reduces the direct detrimental effect of drying on microorganisms and, indirectly support microbial growth by prolonging the period during which nutrients could be exuded (Waggoner, 1965). Cultural techniques commonly employed in the study of the phylloplane however, do not provide information

relating to topographic variation of the microflora. In fact, a study of the phylloplane not accompanied by the direct observation techniques could lead to erroneous conclusions regarding fungi that are active in this environment.

Correlation exhibited by the microbial populations on leaf surfaces with weather parameters provides an insight into the population variations, and explains the unpredictability and variation found in population levels and composition. Previous attempts to explain seasonal variations in leaf surface microbial populations have been speculative. Last (1955) attributed the summer increase of *Sporobolomyces* to increased leaf age, temperature and air movement. Pennycook and Newhook (1983) considered daily duration and intensity of insolation as causative factors. Hayes (1981, 1982) first attempted to correlate statistically climatic regimes with phylloplane microbial populations, though with negative results. Results presented here are supportive of his suggestion that more remote meteorological events may not be without critical influence. The meteorological factors exhibiting correlation here are the average (temperature and relative humidity) and total (rainfall) values for the fortnight preceding the date of sampling. The effect on *E. nigrum* could be through the increase in concentration of these spores in the aerial microflora. The correlation exhibited by the total microbial population is due to *E. nigrum* which constitutes nearly 80% of the population. Lack of correlation with the established fungus *C. cladosporioides* and the yeasts could be due to the inconsistencies in isolating these populations described above.

These results suggest that the leaf surface as an ecological niche does not harbour a specialized microbial population similar to those of litter, soil and water, but acts as a refuge for numerous airborne fungal propagules. Studies aimed at an accurate image of this environment should employ a variety of methods of observation. Leaf surface microflora appear to be dependant on weather parameters for successful establishment.

Chapter 3

Phylloplane microflora of tea (*Camellia sinensis*)

3.1. Introduction

The potential significance of the saprophytic microflora to processes leading to leaf senescence and their promise as biological control agents of foliar pathogens have provided the incentives for the study of the microbiology of the phylloplane. The phylloplane microflora of plant species in which the leaf constitutes the economic yield assume special importance due to effects on the quality of the final product. the microflora inhabiting the tobacco phylloplane has been investigated (Norse, 1972; Spurr and Welty, 1975) chiefly because of the potential antagonistic effects of the abundant species *Cladosporium herbarum* on the pathogenic fungus *Alternaria alternata*, rather than the effects of the microflora on the quality of the processed leaf. Tea is the only other processed leaf (legally) traded internationally, and a hypothesis regarding the effect of the phylloplane yeasts on tea aroma has been propounded in this dissertation. The successful colonization of the leaf surface is a prerequisite for the envisaged role of the phylloplane yeasts in tea aroma biogenesis (section 1.4). Factors that govern colonization of green leaf surfaces by microorganisms will therefore be of critical importance.

The factors of the environment are by far the most important determinants of the composition and the size of a phylloplane microflora. Such effects operate through two main pathways: a) by determining the nature of the aerial microflora which is the primary source of inoculum and b) by influencing the germination and growth of microbial propagules deposited on leaf surfaces. It has been claimed that the aerial microflora is the single most important source of inoculum for the phylloplane (Dickinson, 1976). The leaf acts as a landing site for various microorganisms in the air microflora (Gregory, 1971; Hudson, 1971; Pady, 1971; Pugh and Buckley, 1971) . Gregory (1973) confirmed that the number of spores deposited on the leaf surface was proportionate to the number of spores in the air spora. The size and the composition of the aerial microflora is determined by the production of fungal propagules elsewhere, their release into the atmosphere and survival in the environment. All these processes are influenced by climatic factors. The influence

of meteorological conditions on changes in the atmospheric fungal spore concentration has been investigated by a number of workers (Hirst, 1953; Kramer *et al.*, 1964; Pady *et al.*, 1969) . Lacey (1962) reported that local environment was important in determining the concentration and composition of air spora in the atmosphere at a given location. Amongst the environmental factors, humidity and temperature have been identified as the meteorological factors exerting the most profound effect on the release and dispersal of air spora (Pugh and Mulder, 1971). Long and Kramer (1972) reported variations in airspora that reflected the season, environmental conditions and time of day. In a study of airborne yeasts in Amristrar, India, Sandhu and Waraich (1981) recorded small numbers of *Candida, Rhodotorula* and *Torulopsis* during winter months. The environmental conditions under which a plant grows will thus partially determine the characteristics of the phylloplane microflora through its effect on the aerial microflora.

The factors of the environment also influence the establishment of the microflora either through direct effects on the microorganisms, or through the effects on the chemical environment of leaf surfaces. Relative humidity is generally regarded as a major environmental factor that influences the success of microbial colonization of leaves (Dickinson, 1967, 1971; Dickinson and O'Donnell, 1977). Organic and inorganic materials, originating within the plant and leaching into water in contact with leaf surfaces, accumulate on leaf surfaces influencing microbial growth. Rainfall and its pH, relative humidity and dew formation have been reported to affect the accumulation of leached substances on leaf surfaces (Godfrey, 1976). The presence of gallic acid in dew on the leaves of *Acer platanoides* has been correlated with the low incidence of *Cladosporium herbarum* on these leaves (Dix, 1974).

An analysis of the phylloplane microflora of *Photinia glabra* and the factors that govern its composition and temporal variation has been presented (Chapter 2). This study revealed that the microbial population on the leaf surface on a particular sampling date was closely correlated with the weather conditions during the preceding fortnight. This evidence suggests that the microflora on *Photinia glabra* growing under mild temperate climatic conditions in Canberra could be drastically different from that occurring under tropical climatic conditions on tea in Sri Lanka. Tea cultivation has recently begun in Innisfail, north Queensland, Australia. Situated at a latitude of 17.32°S, this region has a tropical climate that closely resembles the climate in Sri Lanka (latitude 7.0°S). The reported rainfall pattern in Innisfail (Upsher and Griffiths, 1973) also is similar in distribution to that experienced in the tea growing regions of Sri Lanka. Aerial microflora

occurring under tropical climatic regimes in north Queensland (and therefore the phylloplane microflora) could be expected to resemble closely that occurring in Sri Lanka. Apart from an amateur study done by this author (Wickremasinghe, 1982), reports of the studies of tea phylloplane microbiology are not available in literature. A study of the nature of microbial community on tea leaf surfaces is therefore of relevance in the context of the argument presented in this thesis.

3.2. Materials and Methods

Study area: The sampling area was selected from a tea plantation in Innisfail (17.32°S, 146.02°E), north Queensland, Australia. A sampling area containing three subplots was marked out in a mature tea field not being harvested. The three subplots were similar in aspect, tree age, height and density and were treated as statistical replicates for the tests.

Methods of study: Distance of the sampling site from the laboratory (3300 km) limited the choice of methods of study that could be employed. Study of the tea phylloplane was done using the leaf print technique, the only method that could be employed in the field. It was decided to increase the number of samples to improve the reliability of data.

Sampling of the aerial microflora: Air microflora was sampled at the time the tea leaf surface microflora was sampled. The air microflora was measured at 2 m height on all sampling dates. Sampling was affected using the gravity Petri dish method. Exposed plates were incubated under conditions described below and fungi identified.

Study of the microflora colonising the tea leaf surfaces: The phylloplane microflora of tea was sampled on seven sampling dates each in August 1985 and August 1986, during the 'dry season' in north Queensland. The weather conditions resembled those during the tea 'flavour season' in Sri Lanka. Leaves in different physiological stages were selected for the sampling of surface microflora, from six tea bushes in each of the three subplots. The first, and second leaf from the shoot apex and a mature 'maintenance' leaf from a height of 60 cm above ground were sampled. With a view to study fungal successions, the microflora on the litter were also sampled. In each case, the adaxial surfaces of three individual leaves were pressed firmly on to the surfaces of pre-poured solid culture medium (PDA). To avoid bacterial contamination of the culture medium due to the transportation of plates and work under field conditions, streptomycin sulphate was added (100 mg/L) to the culture medium after autoclaving. A second set of leaves were used to

study the abaxial surface microflora. Inoculated Petri plates were incubated in a closed metal container, at room temperature which averaged 24°C (± 1°C) during the 5 d incubation period. The majority of fungi, which were common aerial contaminants, could be easily identified by colony characteristics, pigmentation of the medium and characteristics of sporulation. In cases where doubts existed, colonies were asceptically subcultured and brought to the laboratory for microscopic examination.

Statistical analysis: The data collected during the two seasons were pooled for the analysis. The total number of colonies of filamentous fungi and the yeasts recorded on the upper surface of the leaves of different degrees of maturity on seven sampling dates on the three plots were subjected to an analysis of variance. The percentage occurrence of each fungus on the upper and lower surfaces of the first, second, mature and litter leaves was calculated using pooled data of both seasons. A comparison of the upper and lower surface microflora was not attempted since the limitations in the microbial sampling technique made it unsuitable for such a study (Chapter 2). Statistical analysis was done using the Genstat statistical package.

3.3. Results

Aerial microflora: During the sampling, 43 genera of fungi belonging to the families Moniliales, Melancoliales, Sphaeropsidales and classes *Ascomycetes* and *Phycomycetes* were encountered. Table 3-1 shows the average numbers of major genera of filamentous fungi and yeasts recorded during the two seasons on Petri plates exposed to air for 3 min. *Cladosporium cladosporioides* constituted approximately 30% of the mycelial fungal propagules recorded on every sampling date. Though significant numbers of *Epicoccum*, *Nigrospora* and *Monilia* were present during the sampling period, all other major genera collectively accounted for only 30% of the total microbial population. The fungi showed no obvious frequency pattern. When seasonal mean spore concentrations were compared, no significant differences were found between the average number of propagules recorded in the two seasons. Daily means compiled from samples taken during each season indicated trends similar to those from seasonal data. The yeast populations in the aerial microflora exhibited larger variation than the mycelial fungi. The yeasts were predominantly *Rhodotorula glutinis* and *Cryptococcus* spp., each constituting 16-17% of the total microbial population. *Aureobasidium pullulans* was present in significant numbers on some sampling dates. The yeast numbers did not differ significantly between the two seasons.

	No of colonies								
Fungus	D*1	D2	D3	D4	D5	D6	D7	Total	%
Cladosporium	15	19	17	14	26	20	11	122	32.1
Geotrichum	6	9	14	10	4	2	8	53	13.9
Mucor	1	-	1	2	-	2	4	10	2.6
Penicillium	4	6	1	3	4	1	3	22	5.7
Trichoderma	-	-	-	-	1	1	-	2	0.5
Fusarium	2	6	-	1	2	2	-	13	3.4
Nigrospora	3	1	2	1	-	-	1	8	2.1
Epicoccum	-	-	-	1	1	-	-	2	0.5
Curvularia	2	-	-	2	3	-	-	7	1.8
Yeasts									
Rhodotorula	6	4	11	13	9	7	12	62	16.3
Cryptococcus	14	12	9	4	8	13	6	66	17.3
Aureobasidium	2	6	-	2	1	3	-	13	3.4

* = Date of sampling

Table 3-1: Composition of the aerial microflora over a tea plantation in north Queensland. (Pooled data for Aug. 1985 and Aug. 1986)

Leaf surface microflora: The microflora observed on the upper and lower surfaces of the leaves of *Camellia sinensis* during the two seasons were similar and consisted mainly of the fungi *Cladosporium cladosporioides* and the yeasts *Rhodotorula glutinis* and *Cryptococcus* spp. The surfaces of the younger leaves appeared to harbour a less varied microflora dominated by the yeasts, while the numbers and the variety of mycelial fungi increased with increasing leaf age. The common aerial contaminant fungi *Fusarium, Nigrospora,* and *Mucor* were not recorded in any of the first leaves sampled during 1985 and 1986, while *Epicoccum nigrum* was recorded on the upper surfaces of 10% of the samples. These species were present in increasing frequency on the surfaces of mature leaves and litter. As shown by the Table 3-2, the yeast *Rhodotorula* was present on 85% of the first leaves sampled during 1985 and 1986. This high percentage gradually declined to 20-30% in the mature leaf and the yeast was absent in the litter. A parallel increase in the numbers of mycelial fungi, led by *C. cladosporioides*, could be observed to accompany leaf maturity. The variety of mycelial fungi and the numbers of each fungi increased dramatically from the second leaf to the mature leaf. While *C. cladosporioides* appeared on every mature leaf examined during the experiment, the percentage occurrence of other mycelial fungi (*Epicoccum, Geotrichum, Nigrospora, Mucor*) recorded a significant increase with leaf age. As revealed by the Table 3-3, the differences among the leaves of different degrees of maturity were highly significant ($P<.005$). Differences relating to date of sampling were non-significant. Table 3-4 show the analysis of variation of the yeast populations on tea leaf surfaces. The position of the leaf was found to be the factor which contributes most to the variation.

3.4. Discussion

The microflora on tea leaf surfaces in north Queensland exhibits significant qualitative and quantitative differences to the microflora observed on the leaf surfaces of *Photinia glabra* in Canberra. This can be attributed to the differences between the aerial microflora at the two locations rather than host specificity of the phylloplane microflora. The preponderance of *Epicoccum nigrum* and *C. cladosporioides* on leaf surfaces under different climatic conditions is a conspicuous effect of the aerial microflora on the phylloplane. However, the often emphasized influence of the aerial microflora on the composition of the phylloplane microflora has not been articulated. Qualitative and quantitative influences of the aerial microflora on phylloplane microflora need to be distinguished. Present results and other reports suggest that the aerial microflora exerts a

	First leaf		Second leaf		Mature leaf		Litter	
	u*	l#	u	l	u	l	u	l
Cladosporium	22.2	31.3	68.2	100	100	100	100	100
Fusarium	-	-	14.2	6.34	23.8	9.5	26.9	36.5
Penicillium	14.2	9.5	19.0	22.2	52.3	30.1	66.4	92.0
Nigrospora	-	-	9.5	-	25.3	9.5	38.0	15.8
Mucor	-	-	1.58	3.1	9.5	6.3	19.0	9.5
Epicoccum	10.1	-	10.6	-	15.2	9.0	32.5	29.0
Rhodotorula	85.7	68.2	43.7	31.0	36.4	16.1	-	-
Cryptococcus	62.2	33.3	16.5	11.7	9.5	12.6	-	-

* = Upper leaf surface, # = Lower leaf surface

Table 3-2: Percentage frequency of occurrence of fungi on tea leaves as indicated by leaf print technique (pooled data for Aug. 1985 and Aug. 1986)

Source of variation	DF	SS	MS	VR
Position of leaf	2	372282.0	186141.0	458.4^{***}
Date of sampling	6	678.1	113.0	0.2^{ns}
Error	54	17054.7	406.1	
Total	62	392119.7	6324.5	

DF = Degrees of freedom, SS = Total sum of squares, MS = Mean sum of squares, VR = Variance ratio (F value) *** = Significant at P<0.005, ns = Non-significant

Table 3-3: Analysis of variance of the filamentous fungal populations on tea leaf surfaces

Source of variation	DF	SS	MS	VR
Position of leaf	2	113631.5	56815.3	212.2^{***}
Date of sampling	6	3059.0	509.3	1.9^{ns}
Error	42	11245.3	267.7	
Total	62	132861.7	2142.9	

DF = degrees of freedom, SS = Total sum of squares, MS = Mean sum of squares, VR = Variance ratio (F value), *** = Significant at P<0.005, ns = Non-significant

Table 3-4: Analysis of variance of the yeast populations on tea leaf surfaces

clear qualitative effect on the phylloplane microflora, though results to the contrary have been reported (Lamb and Brown, 1970). A quantitative realtionship may be more difficult to establish due to the isolation of propagules from established microbial populations on the leaf surface.

The composition of the aerial microflora at Innisfail, north Queensland appears similar to those recorded at many other tropical locations. The widespread occurrence of *Cladosporium* in tropical areas with distinct 'wet' and 'dry' seasons has been previously observed (Dransfield, 1966; Turner, 1966) . Relatively high population levels of *Nigrospora* and *Geotrichum* have been observed during August-October in a three year survey of the aerial microflora in Innisfail (Upsher and Griffiths, 1973). In this study, different fungi have been found to predominate the aerial microflora during different times of the year. Thus the microflora recorded in the present study appears to be that characteristic of the hotter part of the dry season. This points to a time specific nature of observations made in similar studies. Further, such variation shows that the aerial microflora in a given location undergoes considerable variation reflecting the effects of prevailing weather conditions on sporulation and transport of fungal propagules. Such effects may account for the irregular occurrence of the minor components of the aerial microflora.

The microflora on leaf surfaces exhibits a broad similarity to that observed in the aerial microflora. However, the relative numbers of each fungus on the leaf surface bear no quantitative relationship to the aerial microflora. This was particularly the case with the younger leaves (first and the second leaves from shoot apex) which were colonised predominantly by the yeasts. The yeasts were minor components of the aerial microflora. This probably suggests that the emerging bud and the leaves carry a microbial population which originates from other sources than the aerial microflora. The composition of the fungal population on mature leaves bear close qualitative resemblance to that of the aerial fungal flora. The large increase in the populations of the yeasts can be attributed to subsequent multiplication on the leaf surface, upon inoculation from the atmosphere. The technique of isolation used in this study, leaf print technique, does not discriminate between the microbial propagules that are casual itinerants and those that are true inhabitants of the leaf surface. Therefore the populations observed on leaf surfaces can be regarded as consisting of both these components. The microflora on the litter becomes even more complex due to the diversity of sources that could contribute to this microflora. Numerous fauna that live in the eco-system within the tea plantation are probably

responsible for the relatively high number of soil fungi (*Fusarium, Trichoderma*) observed on litter. Leaf maturity appears to be accompanied by drastic changes in the composition of the phylloplane microflora, with mycelial fungi begining to dominate. Changes in the chemical environment on leaf surfaces following leaf maturity have been held responsible for this general trend in phylloplane microbial populations (Dickinson, 1976). Mycelial fungi seem to be benefitted more by the changing conditions than the yeasts. A proportionate reduction of yeasts in the litter leaf which is thickly covered with fungal mycelia provides further evidence to this effect. Lack of significant differences between the adaxial and abaxial microflora on many of the leaves sampled confirms the observation made in the study of the leaf surfaces of *Photinia glabra* that leaf print technique is an unsatisfactory technique for the study of such differences.

Analysis of variance of the populations of the mycelial fungi and the yeasts show that the degree of maturity of the leaf is the only significant factor which contributes to variation. The effect of leaf age on phylloplane microbial populations has often been studied only as a complicating factor of the effect of the advancing season. This experiment however, generates useful data due to the sampling of leaves of different ages simultaneously. Lack of relationship between the fungal flora on the surfaces of younger leaves and the aerial microflora, and the progress of this fungal population with leaf maturity, suggest that the establishment of a substantial phylloplane microflora awaits changes in the leaf surface environment. The maintenance leaf sampled in this experiment was approaching senescence and the tissues on the litter leaf would have already disintegrated. Under such conditions, the nutrients contained in the cells can be assumed to exude profusely to the leaf surface, increasing the availability of nutrients to these microorganisms. The mature leaves, being located well within the canopy or beneath it, are protected from the damaging solar radiation and are maintained in an atmosphere of high relative humidity. The combination of these conditions appear to be favourable for the establishment of a fungal population. The development of a large microflora on the younger leaf is hampered by its relatively short exposure to atmosphere and its higher susceptibility to vagaries of weather conditions, caused by the location of these leaves at the top of the canopy. A lack of correlation between the aerial microflora and the microflora on younger leaves is indicated by the absence of predominant aerial fungi on the surfaces of these leaves. The yeasts that were commonly recorded on the surfaces of young tea leaves were not often found in the aerial microflora. However, the complete transformation of this microflora with advancing leaf age appears to be the norm. Lack of significance of the date of sampling can be

attributed to the relatively short duration of sampling. In a long term analysis of the aerial microflora at a location near the site of this experiment, Upsher and Griffiths (1973) reported the preponderance of individual fungal species over two month periods. Sampling on alternate dates for a period of several weeks at a particular time of the year is thus certain to record the presence of one or few species of fungi with little variation in numbers.

This study reveals the fact that young tea leaves in a tropical climate harbour significant populations of yeasts, mainly *R. glutinis*. In view of this, the hypothesis that the pink yeasts synthesize significant amounts of carotenoids that enhance tea flavour, receives support.

Chapter 4

Effect of osmotic water potential on spore germination and hyphal growth of three phylloplane fungi

4.1. Introduction

As explained in chapter 1 (section 1.4), it is imperative that substantial microbial colonization of the tea leaf surface occurs before any important role could be played by the phylloplane microorganisms in tea aroma biogenesis. The leaf surface is an inhospitable environment for microbial growth (Mishra and Dickinson, 1984). Pugh (1974), in an analysis of strategies in fungal ecology, described the leaf surface as a 'high stress/high disturbance' environment. It is characterized by fluctuations in temperature, available moisture, and relative humidity; intense radiation; relatively low and variable nutrient availability; the presence of antimicrobial chemical compounds exuded by the host (e.g. gallic acid) and the scouring action of wind and rain. The developing microbial community is continuously disrupted by these influences, contributing to erosion and death of microbial cells. Unavailability of water, rather than a shortage of nutrients, unsuitable pH or the presence of inhibitory substances has been identified as the primary factor which restricts the establishment of a microflora on the leaf surface (Rodger and Blakeman, 1984). Extensive fungal colonization of the leaf surface cannot be presupposed under such circumstances. Implied norm of fungal activity on leaf surfaces is one of increased activity during periods of dew formation, and relatively inactive periods during dry environmental conditions.

The energy of water in biological systems is quantified in terms of water potential. Water potential is defined as the energy of water in a system relative to the free energy of a reference pool of pure, free water having a specified mass or volume. The reference state of pure, free water is assigned zero water potential. Water in biological substrates however is subject to forces that lower its potential energy relative to the reference state. The water potential of cells must be less than that of their environment if they are to gain water from the environment (Griffin, 1985). Therefore, to absorb water from a substrate of low water

potential a microbe must perform work in raising the water potential from a negative to a more positive state. The greater the work that must be done by an organism to obtain water, the more negative is the water potential of that substrate (Griffin, 1969). The capacity of microbial cells to adjust to external low water potentials depends among other factors, on the internal solute reserves, temperature and the nature of external solute. Some xerotolerant and xerophilic species are able to withstand extremely low water potentials (-60 MPa), due to their accumulation of compatible solutes (Brown, 1978), which act as osmoregulators or protectors of enzyme activity at these low water potentials. However, when a fungus does not possess this capacity, it suffers cessation of growth and other biosynthetic processes at moderately low water potentials (-15 MPa). When some component of its life cycle or growth is inhibited or adversely affected by a reduction in water potential, the organism is considered to be under water stress (Griffin and Luard, 1979). In this sense, the fungi on leaf surfaces can be considered as being constantly under water stress.

The components of the water potential of plant tissues are solute and matric potentials, cell wall pressure and gravitation. In the context of microorganisms growing on aerial plant surfaces, the solute (osmotic) potential becomes the most important component. Studies on the effects of low water potential (water stress) on spore germination, mycelial growth and survival and sporulation have largely been confined to soilborne plant pathogenic fungi, particularly the genera *Fusarium* (Cook and Christen, 1976; Wearing and Burgess, 1979; Sung and Cook, 1981) and *Phytophthora* (Sommers *et al.*, 1970). Such studies in which the optimum and minimum water potential for each of the above phases of the life cycle have been determined are scarce in the case of phylloplane fungi. Few studies of water relations of phylloplane fungi reported have examined the effects of atmospheric relative humidity by placing whole plants or leaf disks (inoculated with fungi) in humidity controlled environments (Bashi and Fokkema, 1977; Dickinson and O'Donnell, 1977; Dickinson and Bottomley, 1980; Mishra and Dickinson, 1984). However, it has been pointed out that atmospheric humidity is unlikely to reflect closely the conditions encountered by microorganisms on leaf surfaces (Last and Warren, 1972). Considerably higher levels of humidity are maintained in the immediate environment of the leaf surface, described as the boundary layer by Sutton (1953). Humidity at the leaf surface is dependant upon the rate at which water vapour is transferred through the boundary layer (Burrage, 1971). Satisfactory techniques to measure events in the boundary layer are not available at present. Substrate water potential, via its effect on transpiration, determines the levels of humidity in the boundary layer.

The potential of water in biological systems is reduced by the presence of solutes. This phenomenon is employed in experiments on the effects of low water potential in pure culture with solute potential as the variable. Even though they neglect significant interactions that take place in natural environments, such experiments provide important data (Griffin, 1981a). In the present experiment, the effects of low osmotic water potentials of the artificial culture medium (obtained by the addition of solutes), on the spore germination and mycelial growth of three of the commonest phylloplane fungi (*Epicoccum nigrum, Cladosporium cladosporioides, Alternaria alternata*) are examined.

4.2. Materials and Methods

4.2.1. Fungi and their sources

Three fungi were used in these studies. All fungi were isolated from the leaf surfaces of *Photinia glabra* plants growing at Weston Park, Yarrulumla, A. C. T. Selected fungi represent those occurring as mycelia and as spores. Spores of *E. nigrum* were abundant on leaf surfaces at this location throughout the year without achieving significant levels of germination or mycelial growth. The spores of *A. alternata* were found frequently with a higher rate of germination. *Cladosporium cladosporioides* is representative of a fungus which truly colonizes the phylloplane. Actively growing mycelia of this fungus was observed on the abaxial surfaces of the leaf. Fungi were isolated from leaf prints made on PDA and the first generation cultures were used in the experiment. These fungi were saprophytic on green leaves.

4.2.2. Culture medium

The effect of solute water potential on spore germination and mycelial growth was investigated using culture medium osmotically amended to various water potentials by the addition of KCl, Na_2SO_4, glucose or sucrose. Such a range of electrolytic and nonelectrolytic solutes were chosen in keeping with the recommendation of Griffin (1981). Solute concentrations needed to achieve given water potentials at different temperature levels were calculated from a modified van't Hoff relationship (Papendick and Campbell, 1981) using osmotic coefficients of Robinson and Stokes (1959). A range of water potentials from -0.1 to -14 MPa were obtained by the addition of above solutes to a relatively high water potential basal medium (Sommers *et al.*, 1970). The composition of the basal medium and the quantities of solutes added to obtain predetermined water

potentials are given in Appendix A and B respectively. All media were adjusted to pH 7.0 by the addition of 1M NaOH, and autoclaved at 0.8 MPa for 20 min. Flasks were sealed with aluminium foil to minimize water loss. After each medium had cooled to about 40°C it was dispensed at the rate of 15 ml/Petri plate (9 cm diam). Water potentials of the media were checked using a HR-33 Dewpoint microvoltmeter and C-51 sample chamber (Wescor Inc., Logan, UT, U.S.A.). The water potential of the basal medium was -0.12 MPa. Use of such a medium of low nutrient levels and relatively high water potential was preferred in view of speculated interaction between level of nutrition and water potential (Griffin, 1969; Griffin, 1981; Griffin and Luard, 1979).

4.2.3. Spore germination on media of low osmotic water potential

Stock cultures were maintained at 25°C on PDA slants in McCartney bottles. Appropriate weights of solutes were dissolved in sterile distilled water to obtain equal water potentials to those of the solid media. Spores were washed into these solutions. With the help of a haemocytometer, spore concentration was adjusted to approximately 10^3/ml. Aliquots (0.1 ml) of these spore suspensions were spread on membrane filters (0.2 µm pore diam) placed on solidified, osmotically adjusted culture medium in 5 cm diam Petri plates. Spore germination was evaluated at 6 h, 12 h, and 24 h intervals. The total area of the membrane filter was examined under microscope (x 400) and the number of spores germinated expressed as a percentage.

4.2.4. Mycelial growth on media of solute-amended water potential

Three fungi were grown in 9 cm diam Petri plates on basal medium agar until the colony covered approximately 75% of the agar surface. Plates of water potential amended culture medium were inoculated in the centre with an inverted 4 mm diam agar disk cut with a sterile cork borer, from the periphery of the actively growing colony. The inoculated plates were secured with rubber bands, placed in plastic bags and incubated at 10°C, 15°C, 25°C or 30°C. Plates were examined after incubating for 5 d, and 7 d. Five replicate plates were used to measure colony diam. Growth rate was calculated (mm/d) and plotted against water potential.

4.3. Results

4.3.1. Spore germination at low water potentials

Figure 4-1 shows the relationship between water potential and spore germination. Spore germination of the three phylloplane fungi tested followed a similar pattern. In un-amended culture medium, the germ tube initiation takes place in less than 6 h, but this period increases with progressively declining water potentials. The minimum water potential at which spore germination occurs within 6 h is between -2 to -4 MPa. Spores of all fungi achieved 100% spore germination in 12 h at moderately high water potentials. The critical water potential for each fungus varied; -2 MPa for *E. nigrum*, -3 MPa for *C. cladosporioides* and -4 MPa for *A. alternata*. Beyond these water potentials, within the next -1 MPa decline in water potential spore germination reduced by 20-40% in different fungi. After 24 h there was only a marginal improvement of the percentage of germination. At -7 MPa water potential only 20% of the spores of *E. nigrum* and *C. cladosporioides* germinated. *Alternaria alternata* maintained 50% spore germination at this potential.

Spores of *A. alternata* germinated at water potentials down to -5 MPa in less than 6 h. However, at water potentials below this the percentage germination reduced significantly. After 24 h, germination at lower water potentials improved only marginally. Spores of *E. nigrum* germinated at -2 MPa water potential, though after significantly longer period of time. At -0.12 MPa (unamended basal medium water potential) germ tube initiation was observed after 2 h. At -2 MPa this process took 6 h. Under lower water potentials the rate of germination improved slightly in 24 h. When the water potentials approached -8 to -10 MPa, spore germination reduced to zero. Similar trends were observed with the spores of *C. cladosporioides*. However, these spores could achieve 100% germination upto -3 MPa. The improvement of germination with time at low water potential was greatest in *C. cladosporioides*.

4.3.2. Fungal growth in relation to water potential of agar media

The effect of water potential on the growth of all fungi under investigation was similar. Irrespective of the solute used to amend water potentials or the temperature of incubation, growth was stimulated by moderately low water potentials and progressively declined with further lowering water potentials. Beyond this optimum water potential, the growth rate decreased to a near 50% of the maximum at approximately -5 MPa, and measurable

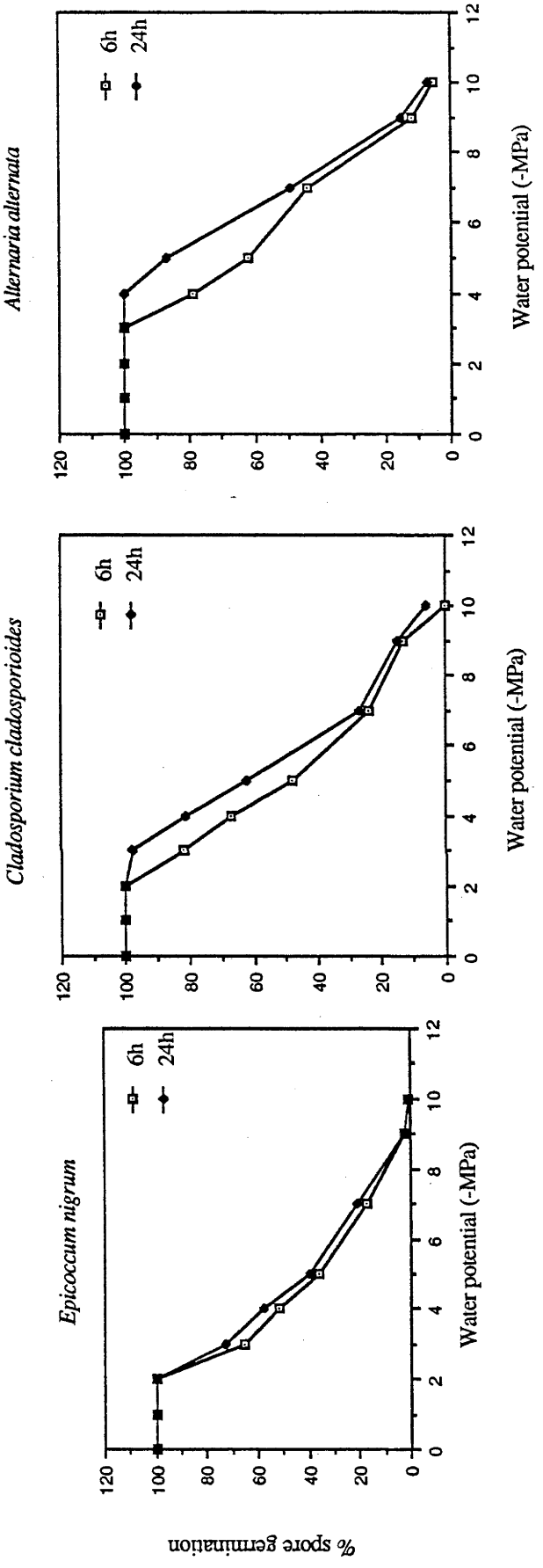


Figure 4-1: Effect of low water potentials on the germination of fungal spores

	WP^1 (- MPa)	WP^2 (-MPa)	WP^3 (-MPa)
Epicoccum nigrum	1 - 2	4 - 5	8 - 10
Cladosporium cladosporioides	1 - 2	3 - 4	8 - 10
Alternaria alternata	1 - 4	4 - 6	10

WP^1 = Water potential at which the highest percentage of spore germination took place WP^2 = Water potential at which 50% of the highest percentage of spores germinated WP^3 = Water potential at which no spore germination took place

Table 4-1: Water potentials at which maximum, 50% maximum and no spore germination took place of the fungi *A. alternata, C. cladosporioides* and *E. nigrum*

growth ceased at -8 to -10 MPa. Table 4-1 presents the water potentials at which maximum, 50% of the maximum and zero growth took place.

Figures 3.2-3.7 illustrate the growth response of the three fungi to low water potentials. At all temperatures and across the solutes, growth stimulation could be observed at water potentials between -0.12 and -3 MPa. With increasing temperature this optimum water potential shifted somewhat towards lower water potentials (Figs. 4-2, 4-4, 4-6). Growth increases were dramatic between -0.12 MPa and -3 MPa for *C. cladosporioides* and *E. nigrum*. This was most apparent in sucrose and KCl amended media. For *E. nigrum* in sucrose amended media the water potential optima were -5, -10 and -4 MPa at 10°C, 15°C, 25°C and 30°C respectively (Figure 4-3). This shift with temperature was common to all fungi. In KCl amended media at 25°C, there was a large increase of growth before the decline. Elevated growth phase in *A. alternata* was less dramatic and was spread over a wider range of water potentials. It was most apparent on KCl amended media (Figure 4-7). For all three fungi, the least value for optimum water potential was in Na_2SO_4 amended medium. At 25°C and 30°C in Na_2SO_4 amended medium, *C. cladosporioides* failed to exhibit increased growth during this phase (Figure 4-5). Growth stimulation in glucose amended media was gradual and spread over a wider range of water potentials.

The rate of decline in growth that followed, also seemed to be solute dependant and was fastest in *A. alternata* (Figure 4-7). The growth decline was sudden in all three fungi beyond -2 to -3 MPa on Na_2SO_4 amended media (Figs. 4-3, 4-5, 4-7). On KCl amended media the effect was prominent only in the case of *C. cladosporioides* (Figure 4-5). The decline spread over a wider range of water potentials on media amended with sugars. The rate of decline also varied with temperature. All fungi experienced the fastest decline of growth rate at 30°C and the slowest decline at 10°C (Figs. 4-2, 4-4, 4-6).

Among the three fungi, there were differences in the overall growth rates, the optimum water potential for maximum growth and the range of water potentials tolerated before undergoing complete cessation of growth. Absolute growth was minimum in *E. nigrum*. *Alternaria alternata* and *C. cladosporioides* achieved colony diameters of 7-8 cm in 7 d at higher water potentials. The effect was similar regardless of the solute used to achieve low water potentials. However, Na_2SO_4 at higher concentrations (at very low water potentials) seemed to cause toxicity. Absolute growth was lower at 10°C and 15°C and reached maximum levels at 25°C. At higher water potentials, all fungi exhibited a faster growth at 30°C, than at 25°C. At lower water potentials this trend reversed, yet exceeded growth

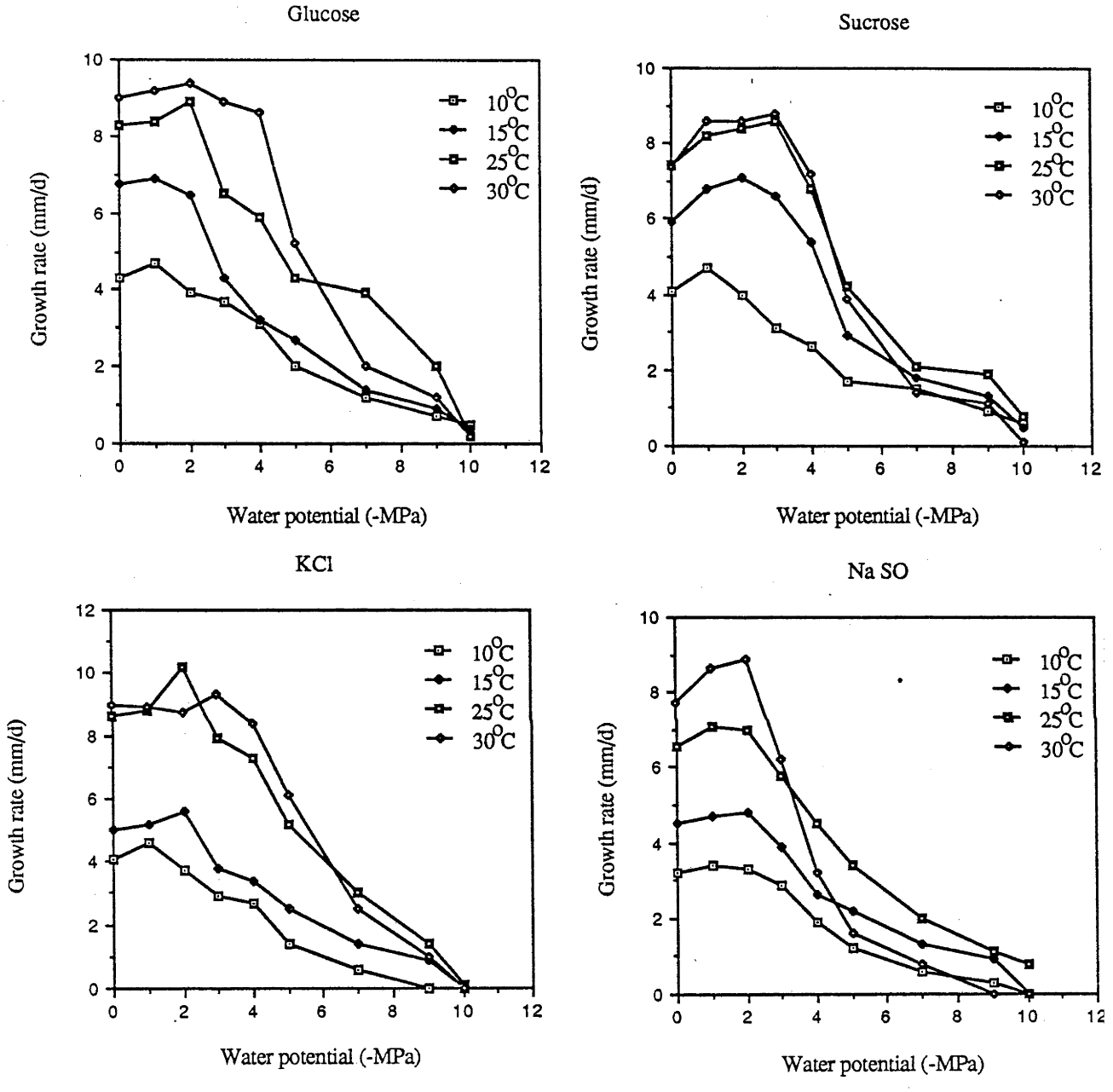


Figure 4-2: Growth rates of *E. nigrum* on culture media of different water potentials obtained with the addition of a. glucose b. sucrose c. KCl d. Na_2So_4

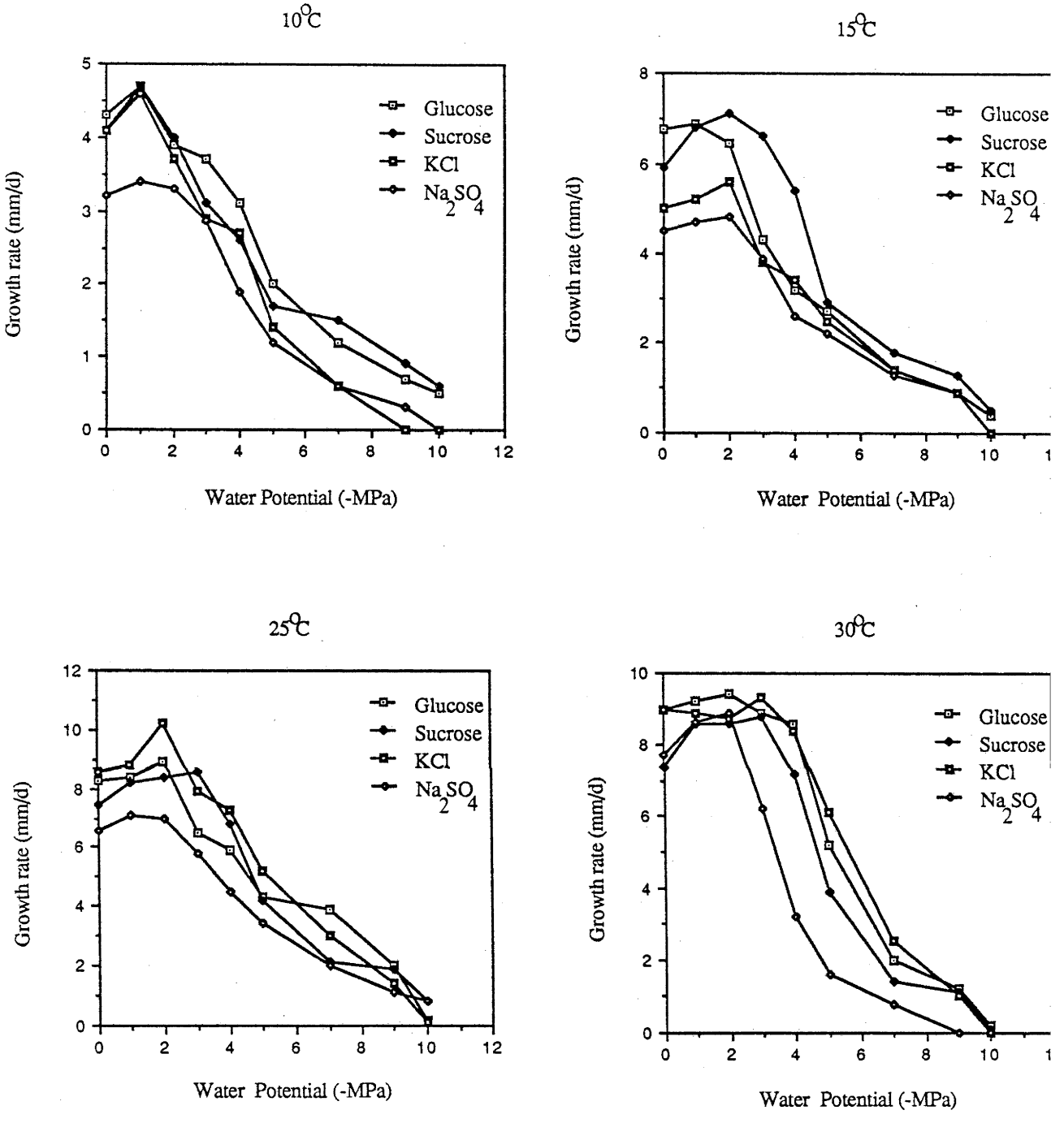


Figure 4-3: Growth rates of *E. nigrum* on culture media of different water potentials at a. 10°C b. 15°C c.25°C d. 30°C

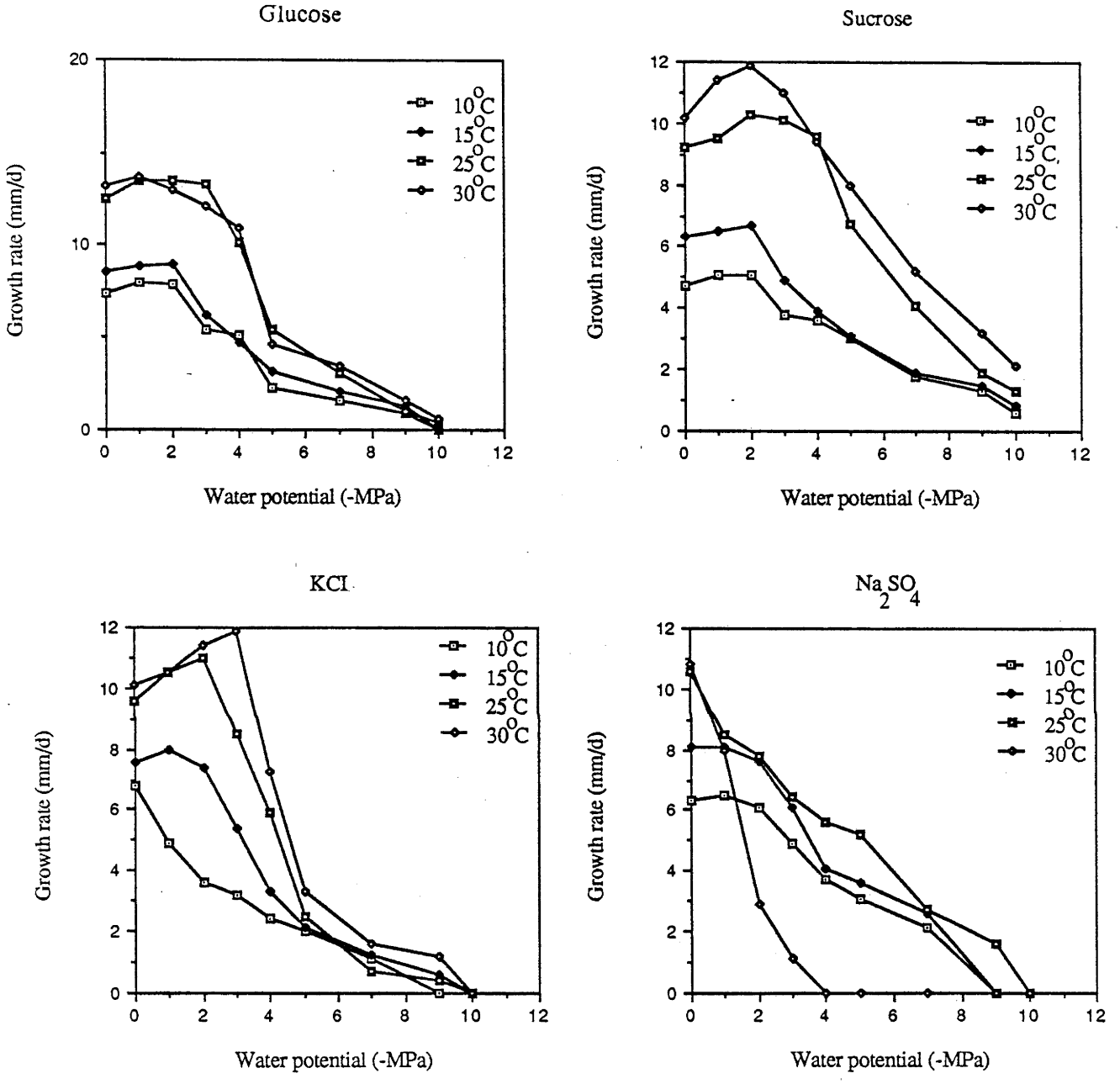


Figure 4-4: Growth rates of *C. cladosporioides* on culture media of different water potentials, obtained with the addition of a. glucose, b. sucrose, c. KCl d. Na_2So_4

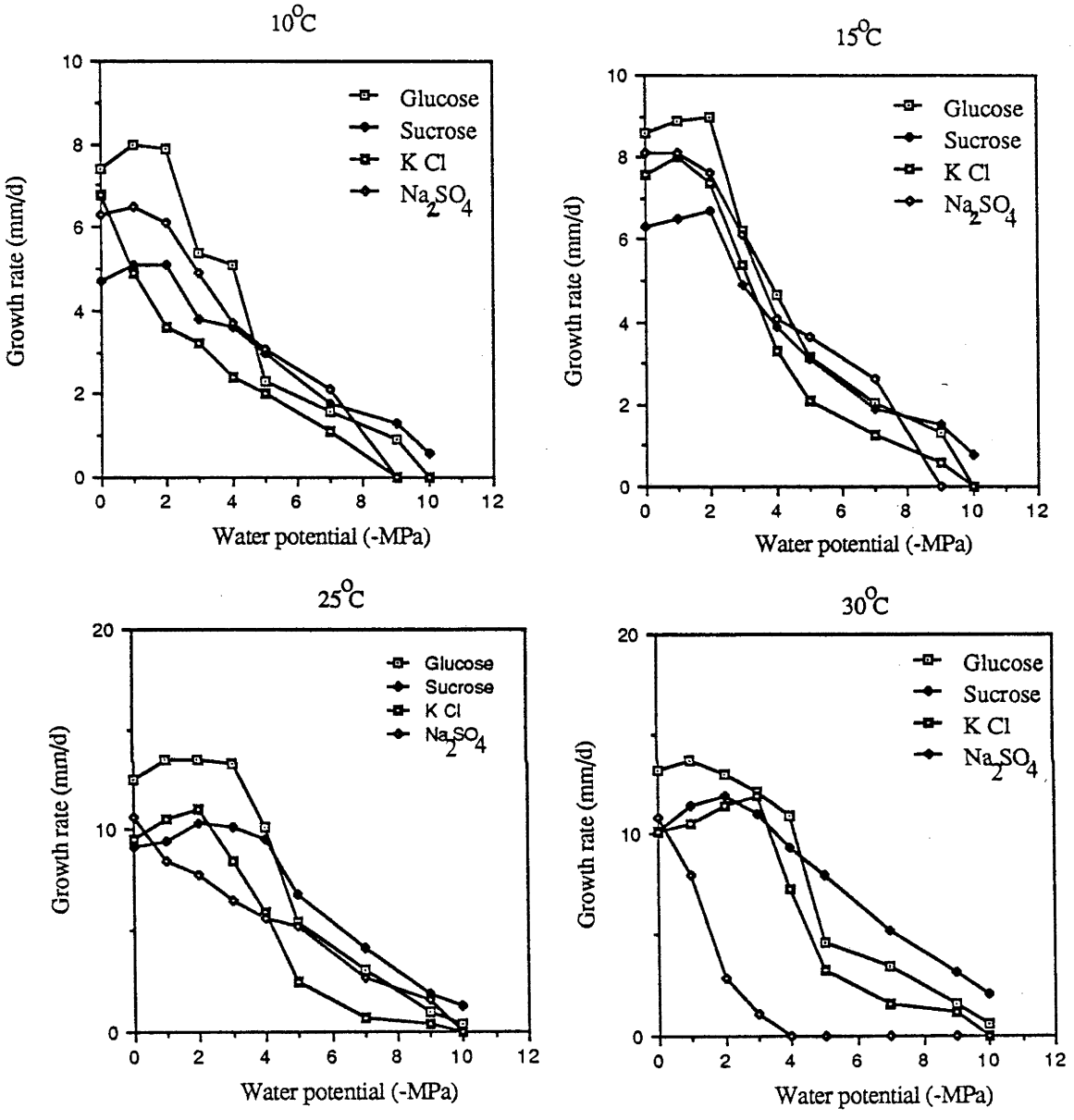


Figure 4-5: Growth rates of *C. cladosporioides* on culture media of different water potentials obtained with the addition of a. Glucose b. Sucrose c. KCl d. Na_2So_4

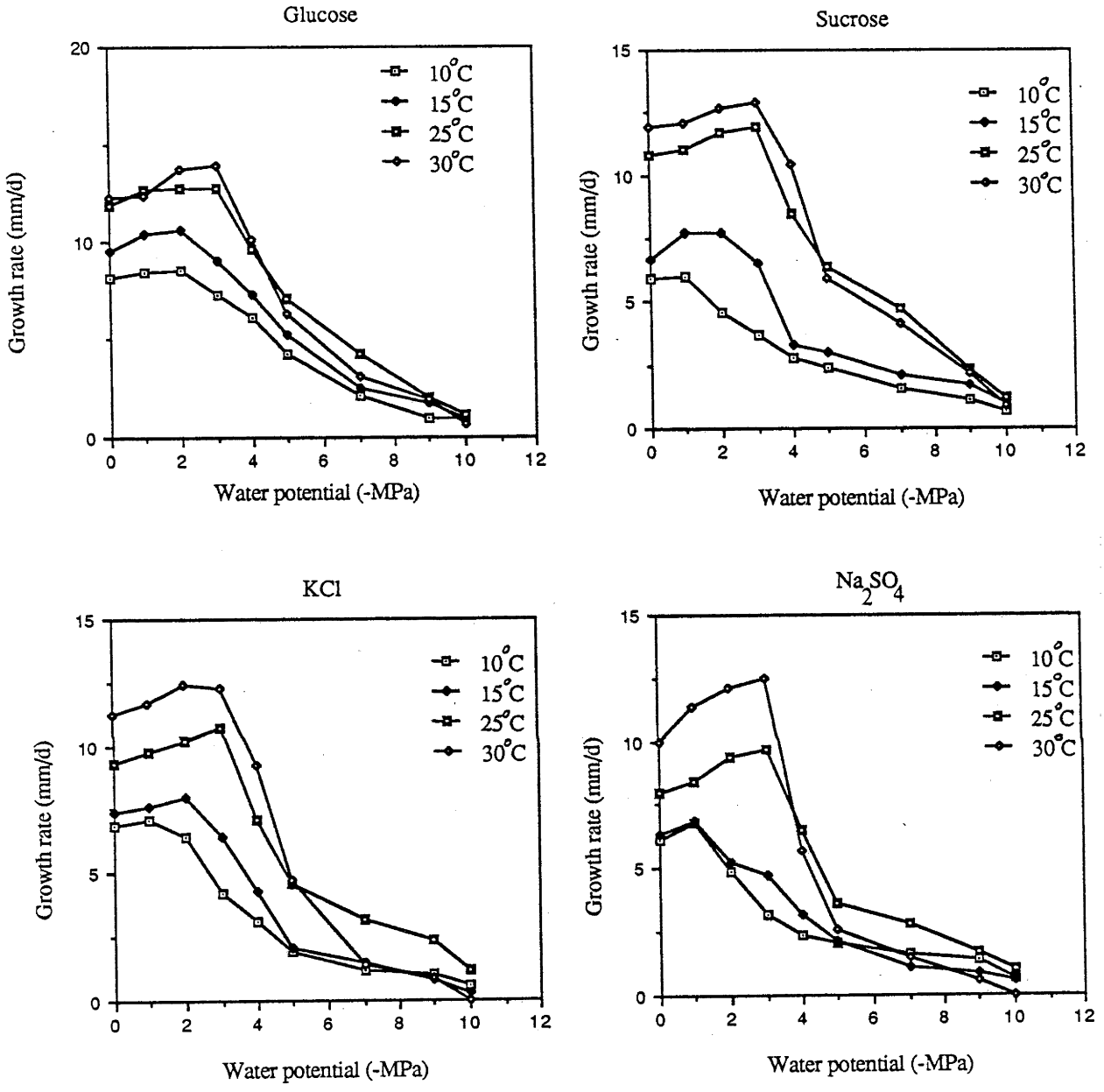


Figure 4-6: Growth rates of *A. alternata* on culture media of different water potentials, obtained with the addition of a. glucose, b. sucrose, c. KCl d. Na_2So_4

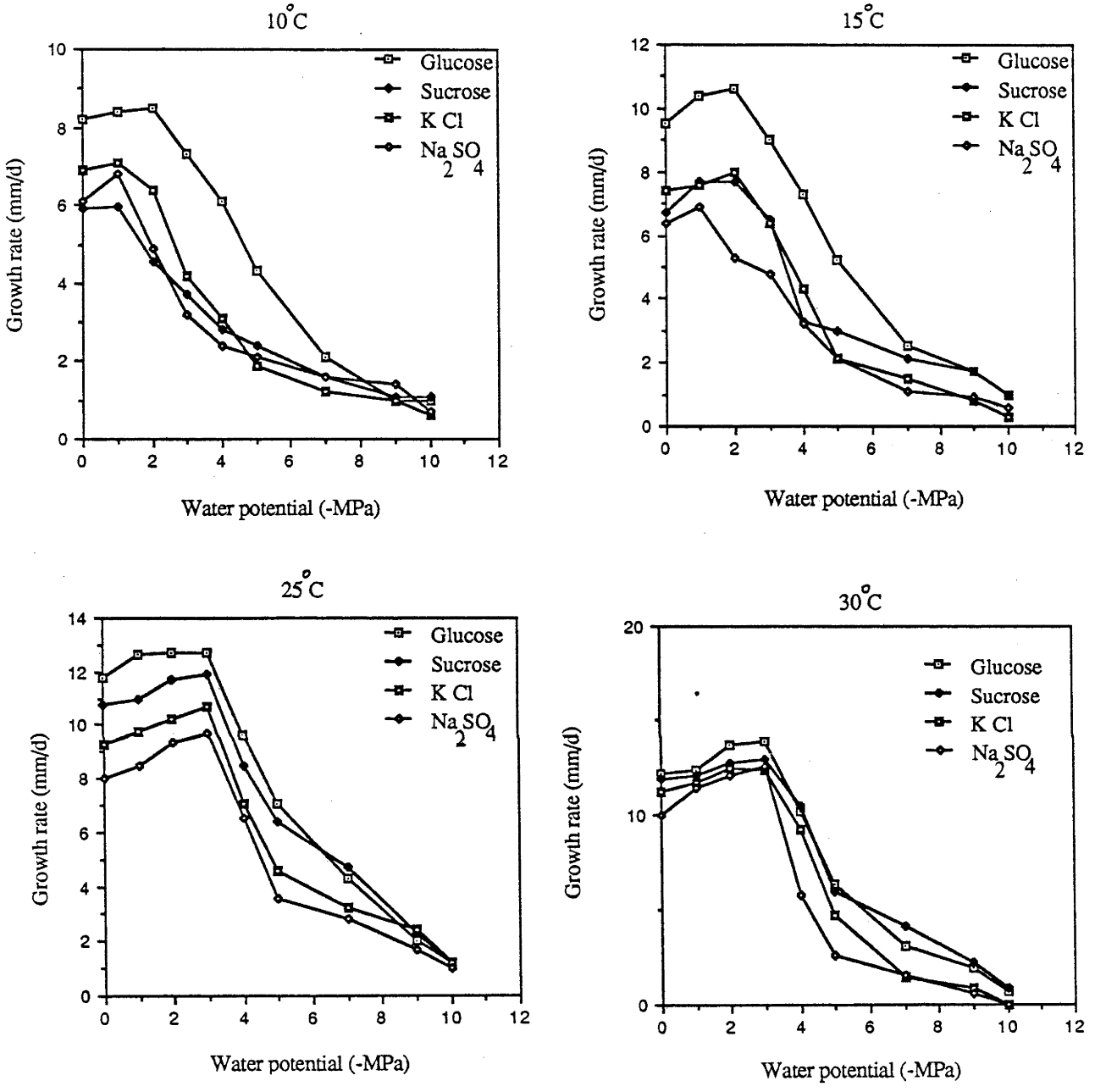


Figure 4-7: Growth rates of *A. alternata* on culture media of different water potentials at a. 10°C, b. 15°C, c. 25°C d. 30°C

levels at 10°C and 15°C. These observations were common to all three fungi. *Cladosporium cladosporioides* growing on media amended with sugars exhibited clear differences in growth rates in the two temperarture groups (Figure 4-4). Growth at higher temperatures was as much as twice that at the lower temperatures. This trend was particularly apparent in media amended with sucrose. On media amended with the two salts (KCl and Na_2SO_4), significantly higher growth did not take place at higher temperatures. In the case of *A. alternata* the temperature effect was clear only on media amended with sucrose (Figure 4-7). *Epicoccum nigrum* colonies on all media grew faster at higher temperatures (Figure 4-2). The decline of growth rate occurring at water potentials beyond the optimum value was faster at higher temperatures. Growth of *A. alternata* was superior on glucose amended media at 10°C and 15°C. Though the growth rates of *C. cladosporioides* and *E. nigrum* were also faster on glucose amended media the effect was not as prominent. Further, the growth of *A. alternata* during accelerating phase (up to -3 MPa) at 25°C shows a clear preference to solutes in the order glucose, sucrose, KCl and Na_2SO_4. This is an exception to the rule that response to low water potential in all three fungi is least dependant of the solute during this range. In the cases of *A. alternata* and *E. nigrum* the growth rate is minimum on media amended with Na_2SO_4. With *C. cladosporioides* KCl occupies this position. Universally, the decline in growth rate beyond the optimum water potential is faster with Na_2SO_4 than with any other solute. *Cladosporium cladosporioides* exhibited a negatively linear relationship with water potential at all temperatures in Na_2SO_4 amended media. The growth stimulating effect of moderately low water potentials was absent. On media amended with non-ionic solutes (glucose and sucrose) the rates of growth decline are similar.

4.3.3. Analysis of Variance

The radial growth data of the three fungi at nine water potentials obtained with four solutes and at four temperatures were subjected to an analysis of variance using the Genstat statistical package. Results are presented in Table 4-2. The analysis showed that water potential, temperature and the solute are all very highly significant ($P<0.001$). The effects of interactions between water potential x temperature, water potential x solute, temperature x solute, water potential x temperature x solute are significant at the same level of probability.

Factor	DF	*Epicoccum nigrum*	*Alternaria alternata*	*Cladosporium cladosporioides*
Water potential	8	17632. 84***	5274.47***	5943.12***
Temperature	3	12194.27***	2502.01***	1597.89***
Solute	3	1814.11***	906.92***	905.12***
WP x Temperature	24	609.42***	152.12***	169.49***
WP x Solute	24	112.89***	44.71***	128.71***
WP x Temp. X Solute	72	276.01***	59.48***	570.19***
Residual	288			

DF = Degrees of freedom, WP = Water potential *** = Significant at P < 0.005

Table 4-2: Values of variance ratio (F) in the analysis of variance of the growth of fungi at low water potentials

4.4. Discussion

Spore germination and hyphal growth of the three fungi investigated, occur at water potentials down to -7 MPa. On the basis of their elevated growth rates at slightly lowered water potentials and cessation of growth at approximately -10 MPa, these fungi may be categorized as Group 3 microorganisms according to the classification of Griffin (1981). Thus, they do not appear to possess specialized capacity to grow at very low water potentials. The optimum water potentials for the growth of these fungi however, seem to be slightly lower than the typical for other fungi in Class 3. The water potential that limits growth is more typical of the fungi in this group.

Dickinson and Bottomley (1980) examined the effect of relative humidity on spore germination of phylloplane fungi, in an experiment in which, the spores of *A. alternata* achieved 100% germination after 14 d incubation at 99.9% r.h. (-0.2 MPa water potential) on glass cover slips, in the absence of nutrients. On green leaves, a substrate relatively rich in nutrients, after 38 d incubation at 70-80% r.h. (-20 to -50 MPa water potential) only 50% of the *A. alternata* spores germinated. However, the authors admitted that the experimental systems employed in these experiments, (coverslips in humidity chambers and whole plants in humidity controlled growth rooms), were less than satisfactory (Dickinson and Bottomley, 1980), especially so because of the imperfectly understood complex water uptake process by germinating spores (Burnett, 1976). Present experiment differs from the above in that the fungal spores have been in more intimate contact with the controlling water potential.

Spore germination of the three fungi are affected similarly by low water potentials. The differences among the lowest water potentials for spore germination are marginal. The larger spores tested, the aleuriospore of *E. nigrum* and conidia of *A. alternata*, suffered greater decline in percentage germination at low water potentials. Due to its higher nutrient reserves (lipids, carbohydrates and proteins), it could generally be expected to utilize those solutes for the maintainance of turgour and osmoregulation. However, such large spores may need free water for hydration (Cook and Duniway, 1981). Inadequate free water rather than low water potentials would have caused the decline in germination. The rate of decline of spore germination at lower potentials is similar except in *A. alternata* in which the rate decreases faster. However, *A. alternata* exhibits the ability for spore germination at the lowest water potential -4 MPa. These data could be complementary to data on survival as those obtained by Dickinson and Bottomley (1980) and Dickinson and O'Donnel (1977).

Pattern of mycelial growth exhibited by these three phylloplane fungi conform to the pattern reported by fungi from comparable environments. The optimum and minimum water potentials for these fungi resemble those of plant pathogenic fungi occurring in dry soils; e.g. *Fusarium roseum* (Cook and Christen, 1976; Wearing and Burgess, 1979; Sung and Cook, 1981). Some soil fungi reported, mainly the species of the genus *Phytopthora* (Sommers *et al.*, 1970) exhibit a greater affinity for higher water potentials. The fungal species reported on here have been investigated in different experimental systems by Diem (1971), Dickinson and O'Donnel (1977), Dickinson and Bottomley (1980) and their results closely resemble present results. The most succesful colonizer of the three fungi, *C. cladosporioides*, however, does not exhibit unusually high tolerance to low water potential. The phenomenon of lower rates of growth of fungi at low water potentials has been attributed to the diversion of energy within the stressed cell, for the production of large amounts of polyols or amino compounds (Griffin, 1981b). Functions of these solutes have been amply documented by Brown (1976, 1978). In the light of this observation it could be deduced that it is the favourable conditions on the abaxial surface of the leaf, rather than any special adaptations of the fungus, that guarantees its success. Lack of increased activity by *E. nigrum* and *A. alternata* on the leaf surface could be attributed to different factors. Spores of *E. nigrum* fail to germinate except when free water is present and the availability of free water on the leaf surface is confined to periods of dew formation or rainfall. Even the highest levels of humidity tested here would not have produced dew formation. Lack of success for *A. alternata* is intriguing in view of its apparent ability to germinate and grow at comparatively lower water potentials. However, the conditions on leaf surfaces are more complex and the presence of germination inhibitors has been reported (Mansfield *et al.*, 1975; Irvine *et al.*, 1978)

Mild stimulation of mycelial growth occurring at moderately low water potentials has been discussed by numerous authors (Griffin, 1981b). The question whether the growth stimulation is a direct effect of water potential or an indirect effect of the solute has often been asked. Scott (1957) postulated that moderate increases in solute concentration in the medium reduce energy requirements to retain solutes, thus increasing the availability of energy for growth. Brown (1964) suggested that the functioning of enzymes is improved by the higher concentration of ions. Absence of any stimulation of growth when the water potentials are controlled by matric mechanisms (Sommers *et al.*, 1970; Adebayo and Harris, 1971; Cook *et al.*, 1972). suggests that the solutes undoubtedly contribute to increased growth at low concentrations (higher water potentials). The solute could well

play a role in the nutrition as well as lowering cell water potential, thus enabling absorption of water. The effect of solutes become apparent again at very low water potentials when high solute concentrations seemingly cause toxicity to fungi. Toxicity is most with Na_2SO_4, particularly at higher temperatures. All three fungi respond similarly to Na_2SO_4 at 30°C, when the growth rate declines rapidly beyond -1 MPa. In addition to the toxicity of the Na^{2+} and $(SO_4)^{--}$ ions, an effect on the pH of the medium could also be suspected.

Other solutes affect the growth of fungi to different extents and in different directions. The effect of sugars generally seems to be beneficial at all temperatures and even at lower water potentials. At 25°C and 30°C *E. nigrum* grew at substantially faster rates on glucose and sucrose amended media than on KCl and Na_2SO_4 amended media. In comparison to glucose, sucrose emerged as the superior solute that can sustain fungal growth even at lower water potentials, particularly at higher temperatures. On sucrose amended medium *A. alternata* and *C. cladosporioides* grew fastest at 25°C and 30°C. Slightly greater growth rates of *Botrytis allii* (Alderman and Lacy, 1984) and three species of *Phytopthora* (Sommers *et al.*, 1970) have been reported on sucrose amended medium. Cook *et al.*, (1972) reported inconsistencies in growth reduction of *Ophiobolus graminis* in sucrose amended growth medium. Fungi included in this category are unlikely to accumulate compatible solutes to any significant extent and the growth stimulation may be caused by improved nutrition. A possible explanation of the preponderance of this effect at higher temperatures is that the enzymes required for the hydrolysis of sucrose are secreted at 25°C-30°C and utilization of sucrose as a source of nutrients takes place. Amongst the solutes, KCl had the least secondary effects on the fungi tested. Similar suggestions have been made by Griffin (1981), and observations reported by Alderman and Lacy, (1984). Analysis of variance quantifies the very significant interactions between water potential, temperature and solute effects. This indicates that the conditions on leaf surfaces are particularly unfavourable for microbial growth.

Results presented here and referred to above point to the need for careful consideration of the solute effects in fungal water relations experiments. The presence of specific solute effects and their dependence on chemical properties of the solute has been pointed out by Griffin (1981) . These results on water relations of common phylloplane fungi confirm the view that most of the fungi encountered in this environment are not specialized inhabitants, but chance itinerants. Water potentials comparable to the optimum for these fungi have been reported on internal tissues of green leaves. Theoretically, the water potential on leaf

surfaces, which are marginally higher than these values, should not inhibit fungal spore germination. However, growth rate in pure culture over a range of water potentials are not usually good indicators of performance at similar potentials in natural systems (Griffin, 1981b). Microbial interactions, presence of inhibitory chemical substances, and sudden and drastic variations in environmental conditions are important determinants of microbial growth on leaf surfaces. Ability to survive dry spells, a trait not evaluated in this experiment, could also be a distinct advantage. Tolerance of moderately low water potentials by these fungi cannot be regarded as an adaptation to life in the leaf surface environment. Resistance to desiccation would better adapt these fungi to survive on the leaf surface, and this aspect needs to be considered in the interpretation of results.

Chapter 5

Effect of environmental conditions on the growth and carotenoids production by *Rhodotorula glutinis*

5.1. Introduction

Pigmented yeasts of the families Cryptococcaceae and Sporobolomycetaceae are reported to be the commonest inhabitants of the phylloplane (Last and Warren, 1972). The biosynthesis of carotenoids is a characteristic of yeasts of the most ubiquitous genera of these two families, *Rhodotorula* and *Sporobolomyces* respectively. The major components of the carotenoids biosynthesized by *R. glutinis* have been identified as β-carotene, γ-carotene, torulene and torularhodin (Fromageot and Tchang, 1938; Bonner *et al.*, 1946; Nakayama et al., 1954) . Biochemical mechanisms involved in the biosynthesis of yeast carotenoids have been comprehensively treated by Goodwin (1972, 1976) and Simpson (1972). The process of carotenoids biosynthesis in yeasts consists of three main steps: a) the formation of the first C-40 carotenoid, phytoene, from isopentyl pyrophosphate, b) the conversion of phytoene to the fully unsaturated acyclic carotenoid neurosporene, and c) the cyclization of neurosporene to γ-carotene. Depending on the environmental conditions, γ-carotene could either cyclize further to give β-carotene, or desaturate to form torulene. Torulene, when synthesized, is oxidized to form torularhodin. By the use of $^{18}CO_2$, it has been shown that oxidation of torulene derives oxygen from the atmosphere (Goodwin, 1972). Torularhodin probably contains oxygen from water (Simpson, 1972). The existence of a control mechanism that govern torulene to torulahodin conversion (that is related to the level of β-carotene) has been suggested (Hayman *et al.*, 1974). Enzymes that catalyse numerous conversions in the biosynthetic pathway have not been characterized (Ruddat and Garber, 1983). Qualitative and quantitative analyses of the carotenoids production have been undertaken (Nakayama *et al.*, 1954; Peterson *et al.*, 1958; Simpson *et al.*, 1964; Vaskivnyuk, 1985). Available evidence suggests that successful growth of yeasts is a prerequisite for carotenogenesis. The amount of carotenoids synthesized depends on the availability of excess carbohydrates after growth has been completed (Goodwin, 1976).

Environmental conditions which affect growth of yeasts could therefore be expected to exert indirect, yet decisive influence on the biosynthesis of carotenoids. Temperature, available water and light, being either limiting or excessive environmental parameters for microbial growth on leaf surfaces, may determine the quality and quantity of the carotenoids biosynthesized.

Yeasts appear to prefer a lower temperature range for growth than bacteria and fungi. Most rapid growth of yeasts occur in the range of 20°C-30°C, with lethal temperatures for vegetative cells occurring between 50°C-60°C (Stokes, 1971). The effect of temperature on carotenoids biosynthesis has been investigated, yielding contrasting results. In *R. sanneiei*, temperature changes between 14°C and 28°C have been reported to have no qualitative effect on pigmentation (Fromageot and Tchang, 1938). At 5°C *R. glutinis* forms a larger amount of β-carotene and γ-carotene than torulene and torularhodin, with the contents reversing at 25°C, suggesting a profound qualitative effect of temperature on carotenogenesis (Nakayama *et al.*, 1954). Simpson *et al.* (1964) have found higher contents of β-carotene at 5°C, and higher contents of torulene and torularhodin at 25°C. There has been no change in the content of γ-carotene.

Reported water potential optima for the growth of yeasts seem considerably higher than those for fungi. While fungi continue to maintain appreciable rates of growth up to water potentials of -30 MPa, the water potential threshold for the growth of yeasts is reported to be approximately -18 MPa (Do-Carmo Sousa, 1969). Exceptions to this limit however, are the xerotolerant yeasts which grow upto -70 MPa, though at greatly reduced rates of growth (Anand and Brown, 1968). While many factors influence the effects of water on growth of osmophilic yeasts, their growth is directly proportional to water activity (Tilbury, 1980). Reports on the effect of water potential on carotenoids biosynthesis are lacking.

Numerous reports on the effect of light on carotenogenesis are available in literature. Effect of light on *R. rubra* and *R. peneus* cultured at 28°C has been simply quantitative (Nakayama *et al.*, 1954). Qualitative changes were observed in *R. gracilis* where the α+β-carotene/torulene ratio changed from 1.67:1 in the dark to 2.29:1 in the light (Simpson *et al.*, 1964).

The recorded optimum water potential and temperature ranges for the growth of yeasts are based on the research on xerotolerant and psycrophilic yeast species that cause spoilage

of preserved foods (Scott, 1957; Anand and Brown, 1968). Growth requirements of these species may not be representative of yeasts from other substrates. The unusual specificity of yeasts in natural habitats is determined by the temperature, nutrients present and other environmental factors, with definite evidence that the maximum temperature for growth is related to the natural environment in which species normally occur (Phaff and Starmer, 1980). This necessitates characterization of yeasts from each individual environment. The present study reports the effects of water potential, temperature, age of culture and illumination on the growth, and qualitative and quantitative aspects of carotenoids biosynthesis by *R. glutinis* isolated from leaf surfaces.

5.2. Materials and Methods

Yeast cultures of *R. glutinis* isolated from the leaf surfaces of *Photinia glabra* were maintained at 25°C on PDA in McCartney bottles. For the inoculation of culture plates, a suspension of yeast cells was obtained in sterile distilled water and the concentration of yeast cells adjusted to 10^3/ml, with the help of a haemocytometer. Aliquots (0.1 ml) of yeast cell suspension were used to inoculate solid culture medium (PDA). In each experiment, at each level of treatment, five plates each were inoculated for the determination of growth weight and analysis of the pigments complex. The experiments were repeated three times.

5.2.1. Effect of water potential and temperature on growth and carotenoids production

The water potentials of the culture medium were altered by the use of sucrose or KCl to obtain water potentials down to -20 MPa (at -2 MPa intervals) according to the procedure described in Chapter 3. Checks of actual water potentials of the medium were made with HR33-Dewpoint Microvoltmeter. Inoculated plates were secured with rubber bands, and incubated in polythene bags for 5 d at 10°C, 15°C, or 25°C. Dry weight determination and the analysis of pigments was carried out after incubating for 5 d.

5.2.2. Effect of age of culture on carotenoids production

Twenty five culture plates (PDA) inoculated with the yeast were secured with rubber bands and incubated at 25°C. Samples of five plates each were removed on 3 d, 5 d, 7 d, 9 d and 14 d after incubation. The determination of total carotenoids contents and torularhodin content was carried out as described below.

5.2.3. Effect of light on carotenoids production

Fifteen culture plates were divided into three groups of five plates each. Cultures to be grown without illumination were wrapped tightly in black polythene, inoculted and incubated at 25°C for 5 d. A set of five plates were inoculated and incubated without illumination for 6 h, and then exposed to light. Another set of five plates were inoculated and wrapped in black polythene after exposing to light under lamina flow for 6 h. A fourth set of five plates were incubated under normal illumination in the incubator.

5.2.4. Determination of growth weight of yeast cell mass

Yeast cells from the five culture plates were carefully scraped with a sterile spatula into a pre-weighed clean watch glass. Any remaining cells were rinsed off with a stream of acetone. After allowing acetone to evaporate, the cell mass was dried at 45°C for 24 h and weight of cells recorded.

5.2.5. Extraction of carotenoids

Yeast cells were scraped off the agar surface with a sterile spatula on to a pre-weighed, sterile watch glass. Remaining yeast cells on the agar surface were washed off with a powerful stream of acetone from a wash bottle. Acetone was later evaporated under a slow stream of nitrogen. The yeast cells were transferred to a mortar and ground vigorously with a pestle after adding 30 ml of acetone (analytical reagent grade). The extract was filtered through a sintered glass funnel (no. 2), and the cell residue was re-extracted with acetone. Three such extractions were usually required to extract all pigments, leaving a white cell mass. Bulked pigment extracts were transferred to a 250 ml separating funnel and 30 ml of n-hexane was added. Distilled water was added (in 10 ml portions) until the acetone-water phase and n-hexane phase clearly separated. Acetone-water phase was drawn off and the pink hexane layer containing all the pigments was washed three times with distilled water to remove all traces of acetone. Clear pigmented hexane layer was filtered through anhydrous Na_2SO_4, to a 100 ml volumetric flask and made to volume. Pigments adsorbed to Na_2SO_4 was rinsed with extra n-hexane. Cell residue was dried at 45°C for 24 h and dry weight of cells recorded.

5.2.6. Quantitative determination of carotenoids

Quantitative determination of the carotenoids was done using a Pye Unicam SP1800 UV spectrophotometer. Prior to the quantification, absorbance of a dilution series of the pigments complex was determined. A linear response was observed, indicating that the absorbance of the pigments complex conforms to the Beer-Lambert law. Absorption spectra of the pigments complex (400 nm-600 nm) in n-hexane were determined against a hexane blank. This was routinely done in order to determine the wavelength of maximum absorption, and absorbance at this wavelength (λ_{max}) was used for the calculations of carotenoids concentration by the following formula.

If x g of the carotenoids in y ml solution gives an absorbance of A at (λ_{max}), then:

$$x = \frac{A \times y}{E^{1\%}_{1cm} \times 100}$$

in which,

x = total carotenoids content (g)
A = absorbance
y = volume of extract (ml)
$E^{1\%}_{1\,cm}$ = specific extinction coefficient (2500)

Carotenoids content was expressed as µg/g dry weight of cells.

5.2.7. Extraction and quantification of torularhodin

Extraction and quantitative determination of torularhodin was done according to the method described by Peterson (1958). This method employs phase separation of the carotenoids complex to measure the xanthophylls content of which the major component in *R. glutinis* is torularhodin. The pigments were first taken up in light petroleum by adding an equal volume of light petroleum (b. p. 40°C-60°C; analytical reagent grade) to the total pigments complex (in hexane) in a 250 ml separating funnel. After gentle mixing, water was added until the two layers separated. A sample from the petroleum ether layer now containing the pigments, was used to determine absorbance at 500 nm. Portions (50 ml) of 90% methanolic potash (0.1 N) were added to the carotenoids in light petroleum, until no colour appeared in the methanolic potash layer. This lower phase containing xanthophylls with two or more free hydroxyl or carbonyl groups was drawn off, and absorbance of the upper phase at 500 nm was determined again. The difference between absorbance before and after extraction with 90% methanolic potash was used to calculate the torularhodin content using the above formula.

5.3.Results

Absorption spectra of the pigments complex (in n-hexane) of the yeast *R. glutinis* grown at 5°C and 25°C are given in Figure 5-1. The peak absorbance of the pigments of the yeasts grown at 5°C was around 454 nm. This peak value shifted to 484 nm in cultures grown at 25°C, indicating a qualitative difference in the pigments complex synthesized at the two temperatures. These absorbance maxima approximate to the recorded values for β-carotene and torulene respectively.

The effects of the factors examined on growth, total carotenoids production and torularhodin fraction were clear. Figures 5-2 and 5-3 show the growth weight of *R. glutinis* at low water potentials in sucrose and KCl amended medium respectively. Low water potentials (below -7 MPa) had a severe negative effect on the growth weights of the yeast. The minimum water potential for growth was -14 MPa. In sucrose amended medium growth stopped at a slightly higher water potential (-10 MPa) than in KCl amended medium. At around -6 MPa water potential, growth weight reduced to 50% of the maximum weight. From this point, growth declined rapidly until it stopped completely at -14 MPa. Growth was generally higher at 25°C than at 10°C or 15°C, regardless of the solute. However, the reduction in growth at higher water potentials was more noticeable at 25°C. At 10°C, the growth reduction was uniform and continued longer. Growth of the yeast in response to low water potentials was similar in KCl and sucrose amended culture medium, except that, in sucrose amended medium at 15°C and 25°C, there was a drastic reduction in growth from -100 to -12 MPa. This effect was not seen in KCl amended medium. In response to the initial -2 MPa decline in water potential, growth reduced remarkably. There was a gradual growth reduction from this water potential, down to -6 MPa beyond which growth declined rapidly. Absolute growth was higher at 25°C than at 10°C or 15°C.

Lower water potentials also affected carotenoids biosynthesis Figures 5-4 and 5-5. The quantity of carotenoids biosynthesized was lower at low water potentials. Water potential at which carotenoids biosynthesis ceased was higher than the potential that caused cessation of growth. Inability to extract appreciable quantities of carotenoids was partly due to the scanty growth of yeasts at these very low water potentials. However, the decline in the carotenoid content was apparent before growth sufferred. At around -6 MPa the quantity of carotenoids synthesized was approximately 50% of the quantity produced in the unamended medium. At 25°C, the synthesis was higher than at 10°C or 15°C,

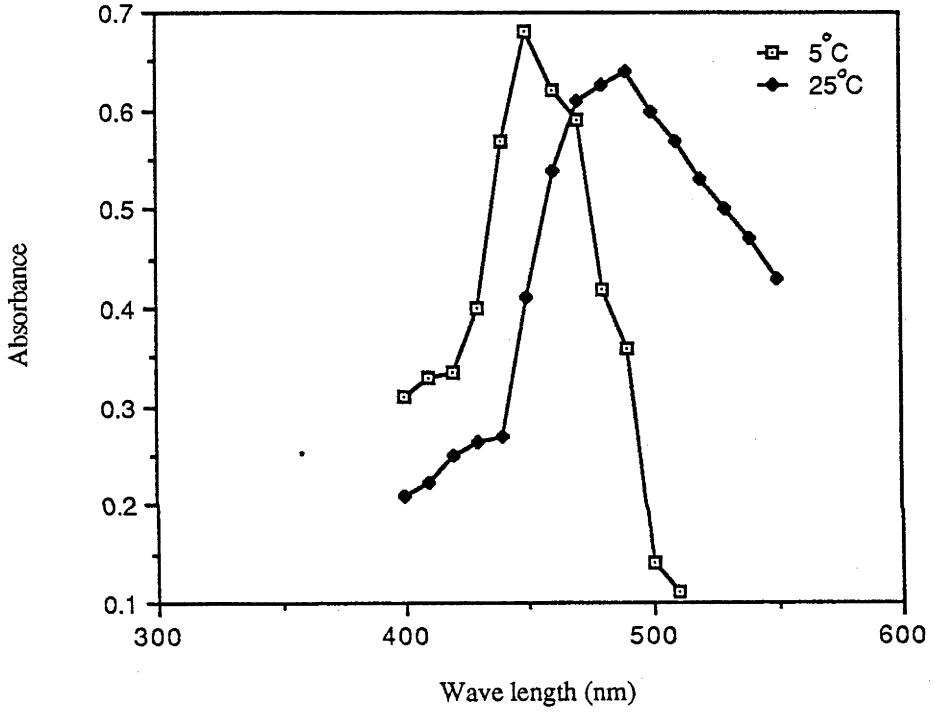


Figure 5-1: Visible light absorption spectrum of the carotenoids of the yeast *R. glutinis*

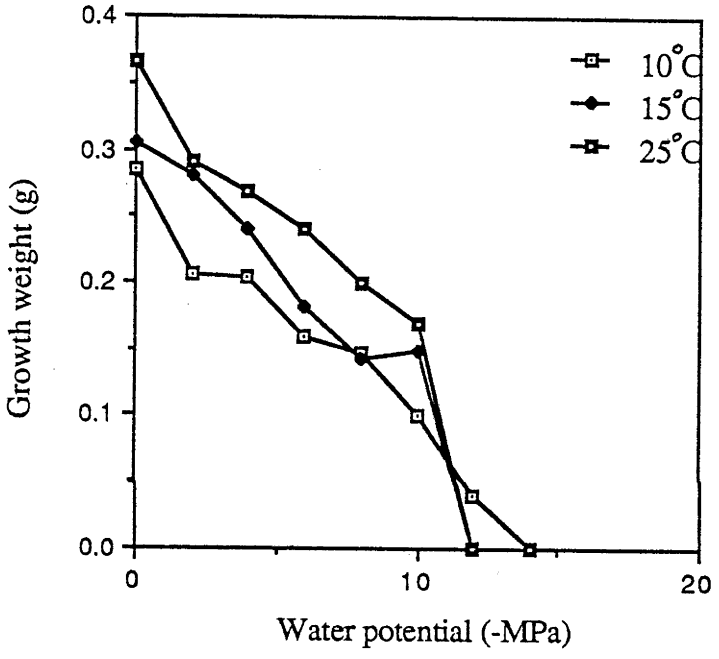


Figure 5-2: Growth of *R. glutinis* at low water potentials obtained with the addition of sucrose

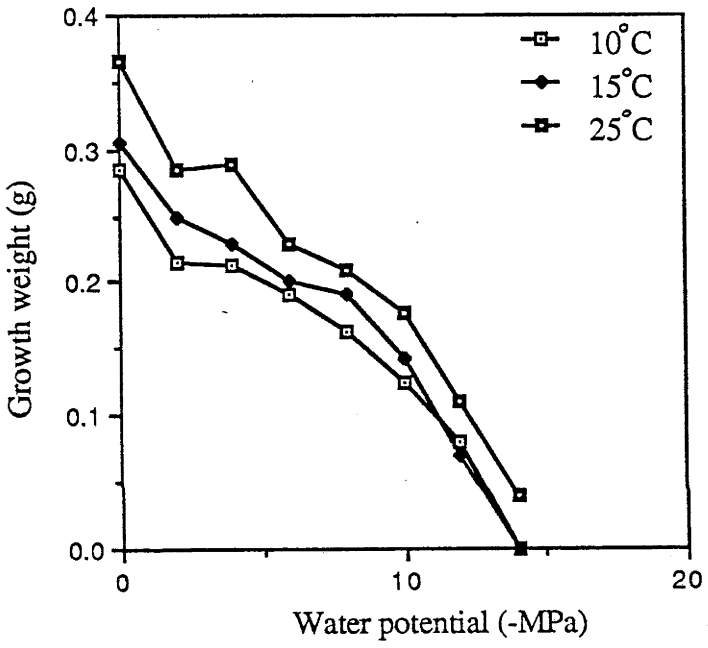


Figure 5-3: Growth of *R. glutinis* at low water potentials obtained with the addition of KCl

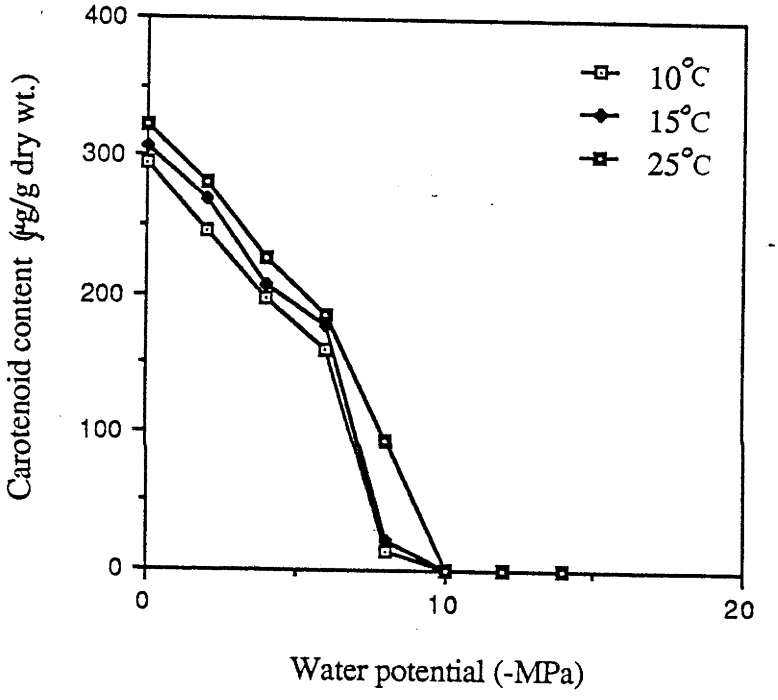


Figure 5-4: Carotenoids production by *R. glutinis* at low water potentials obtained with the addition of sucrose

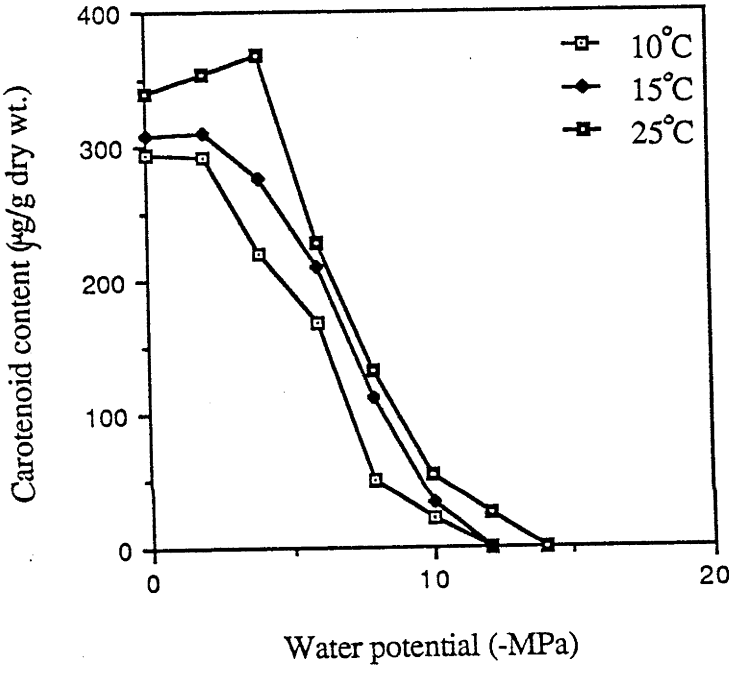


Figure 5-5: Carotenoids production by *R. glutinis* at low water potentials obtained with the addition of KCl

producing measurable quantities of carotenoids at -10 MPa. Carotenoids content beyond -6 MPa was immeasurable at 10°C and 15°C. The rate of decline of the carotenoids contents was similar at all temperatures. In KCl amended growth medium, there was a clear stimulation of carotenoids biosynthesis at -2 MPa at 25°C. This stimulation continued till -4 MPa. The decline in carotenoids synthesis began at -6 MPa water potential and pigment biosynthesis stopped at around -10 MPa. Biosynthesis was marginally higher in KCl amended medium than in sucrose amended medium. In sucrose amended medium, the relative inhibitory effect of the low water potential on carotenoids biosynthesis was higher than in KCl amended medium. The decline started at -4 MPa water potential. Slight stimulation of growth at higher water potentials seen in KCl amended medium was absent in sucrose amended medium.

Torularhodin fraction of the pigments complex declined at progressively declining water potentials. Table 5-1 shows the amounts of total carotenoids and torularhodin produced by the yeast at low water potentials. Torularhodin content (absolute or as a percentage of the total carotenoids) showed little variation until the dramatic decline in the carotenoids content began at around -9 MPa water potential. At water potentials at which, the carotenoids biosynthesis was affected, the torularhodin content (absolute and as a percentage) declined faster. At 25°C, the effect seemed to be less than at 10°C and 15°C. Torularhodin content in KCl amended medium was not significantly higher than the content in sucrose amended medium.

Table 5-2 shows the effect of the age of culture on the biosynthesis of total carotenoids and torularhodin. Age of culture seem to affect both carotenoids content and the torularhodin content. From the third day to the fifth day afetr inoculation, there was a large increase in the total carotenoids content. This increase however, was not reflected by the torularhodin content. Though significant ($P<.05$), the increase in the carotenoids content from the fifth to the seventh day, was not in the same order. From the seventh day, the total carotenoids content exhibited only a marginal increase, whereas the torularhodin content increased steadily at this stage. On the fourteenth day, there was a slight decline in the total carotenoids content. The torularhodin content increased further.

Table 5-3 shows the effect of illumination on the biosynthesis of carotenoids. Total carotenoids content synthesized under illumination, and initial illumination for 6 h (followed by incubation in the dark) do not differ significantly. However, growth without illumination for the first 6 h and the total period of incubation caused a significant

WP (-MPa)	Carotenoid content (µg/g)								
	10°C			15°C			25°C		
	TC[1]	Tor[2]	Tor %[3]	TC	Tor	Tor %	TC	Tor	Tor %
0	294.7	181.4	61.5	307.1	218.8	71.2	321.8	236.4	73.4
2	245.3	112.7	45.9	268.8	172.5	64.1	280.3	214.3	76.4
4	197.3	61.4	31.1	206.4	80.9	39.1	227.1	97.0	42.7
6	159.8	17.5	10.9	177.1	24.4	13.7	184.6	34.9	18.9
8	14.6	-	-	20.9	4.2	20.0	94.3	5.9	6.3

WP= Water potential, 1=Total carotenoids content, 2=Torularhodin content, 3= Torularhodin content as a percentage of the total carotenoids content

Table 5-1: Biosynthesis of total carotenoids complex and torularhodin by *R. glutinis* at low water potentials

Age of culture	Total Carotenoids content (µg/g dry wt.)	Torularhodin content (µg/g dry wt.)	Torularhodin content (% of total)
3 Day	94.2 ± 6.4	63.3 ± 8.6	67.1
5 Day	272.8 ± 5.0	107.8 ± 8.3	39.5
7 Day	322.6 ± 2.9	190.1 ± 6.9	58.9
14 Day	341.9 ± 3.2	284.2 ± 5.7	83.1

Table 5-2: Effect of age of culture on the biosynthesis of total carotenoids and torularhodin by the yeast *R. glutinis*

Illumination condition	Total Carotenoids content (µg/g dry wt.)	Torularhodin content (µg/g dry wt.)	Torularhodin content (% of total)
Continuous	312.2 ± 4.9	211.7 ± 6.5	67.8
6 h illum, (dark)	293.1 ± 5.3	197.3 ± 7.4	67.3
No illumination	262.5 ± 6.8	111.7 ± 8.0	42.5
6 h dark, illum.	281.9 ± 5.8	126.8 ± 10.2	44.9

Table 5-3: Effect of light on the biosynthesis of carotenoids and torularhodin by *R. glutinis*

reduction in the carotenoids content. Torularhodin content followed a similar pattern to the total carotenoids content.

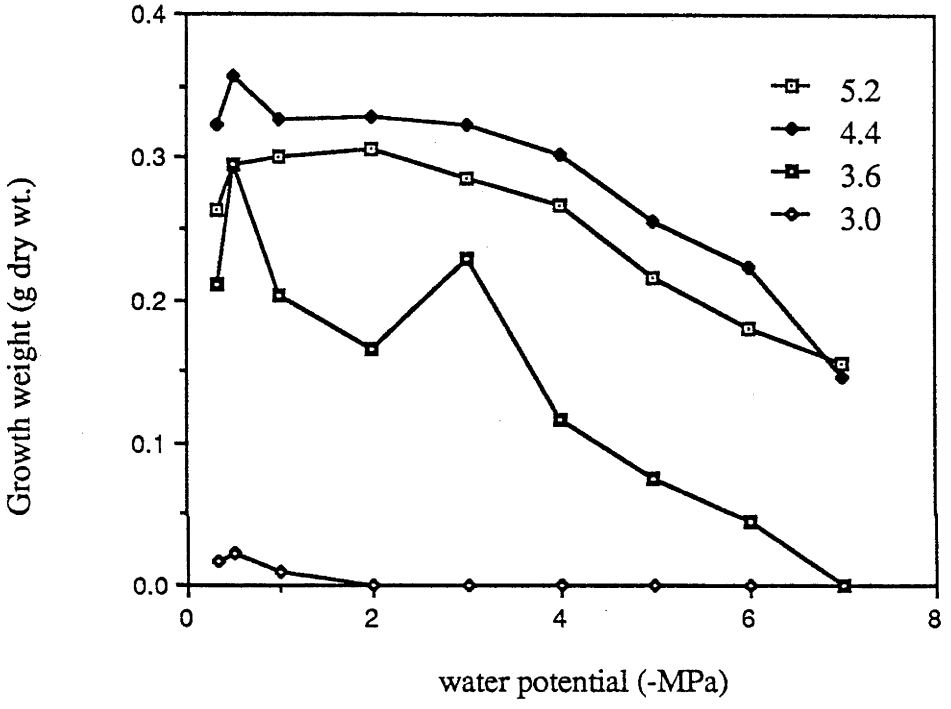


Figure 5-6: Effect of pH on the growth of *R. glutinis*

Factor	DF	MS		F Value	
		KCl amended medium	Sucrose amended medium	KCl amended medium	Sucrose amended medium
Temperature	3	0.00766	0.10310	26.60***	39.77***
Solute	8	0.07151	0.03831	248.23***	147.81***
Temp. x Solute	24	0.00268	0.00577	9.00***	22.27***
Residual	72	0.00028	0.00025		
Total	107	0.00635	0.00462		

DF = Degrees of freedom, MS = Mean sum of squares, *** = Significant ($P < 0.005$)

Table 5-4: Analysis of variance of carotenoids production by *R. glutinis*

5.4. Discussion

These results suggest that the yeast *R. glutinis* is adapted to maintain growth and pigments synthesis at moderately low water potentials. The water potential threshold adequately explains its evident success on leaf surfaces. However, growth at such low water potentials at comparatively high levels of nutrition (in the growth medium) may not indicate a capacity to grow at similar water potentials on leaf surfaces (Griffin 1981, 1983; Griffin and Luard, 1979). Growth decline at low water potentials could be due to similar consequences that affect fungi: solute accumulation necessary to maintain enzyme action at low water potentials draws upon energy, thus depriving the growth process of its requirements. The phenomenon of solute accumulation by yeasts under water stress has been exhaustively treated by Brown (1976, 1978). A higher degree of growth inhibition of yeasts in sucrose amended medium has been reported earlier (Brown, 1976, 1978). This has been attributed to the effect of sucrose on the cell as well as on the enzymes. Cell volumes of nonxerotolerant yeast strains have been reported to change sharply in sucrose (Brown 1976). It has been suggested that sucrose, like many other non electrolytic solutes, exert a direct action on enzyme action by changing the viscosity, pH, ionic strength or dielectric constant of a solution (Brown, 1976). The complex nature of the solute effect on different microorganisms becomes clear by the beneficial effect of sucrose on filamentous fungi, reported in Chapter 3. Sucrose appears to be particularly inhibitive for growth at higher concentrations. This was evident by the rapid growth decline that occurred at -10 MPa water potential. The interactions between the water potential, temperature and the solutes are clear. The analysis of variance (Table 5-4) illustrates the significant effects of the factors as well as interactions among them. At 25^0C, this yeast exhibits a higher capacity to withstand low water potentials. Ability of many microorganisms to tolerate lower water potentials closer to their optimal growth temperatures has been discussed (Griffin, 1981). The maintainance of comparatively higher growth rates in KCl amended medium could probably be due to the nutritive effect of the ions.

The effects of low water potential and temperature on the total carotenoids content and torularhodin content could be explained on the basis of the degree to which the process of biosynthesis is affected by these environmental factors. At higher water potentials tested, the carotenoids content appeared to have only marginally reduced, suggesting that the process continued, though at a lower rate. Proportionate reduction in the torularhodin content (which is the final product of the process), confirms such a proposition. At moderately low water potentials however, torularhodin content (as a percentage of the total

carotenoids content) underwent disproportionate reductions, suggesting that the process takes place at such water potentials and solute concentrations, without completion. This situation may have persisted until the carotenoid biosynthesis ceased at very low water potentials.

The difference in absorption spectra of the carotenoids of the yeasts grown at 5°C and 25°C signifies a qualitative difference in the carotenoids synthesized at these two temperatures. Absorbance maximum characteristic at 5°C (454 nm) indicates that β- and γ-carotene are the major carotenoid synthesized. The absorbance maximum of the pigments complex at 484 nm suggests that the yeast *R. glutinis* accumulates torulene at 25°C. This could probably be due to the incomplete conversion of torulene to torularhodin during the 5 d incubation period. Such an interpretation appears tenable in view of the reports of Nakayama *et al.* (1954) and Simpson *et al.* (1962), who found that β and γ carotene contents were higher at 5°C, but the contents decrease at 25°C with a concomitant increase in the torularhodin content.

Almost identical growth and biosynthesis curves manifest a close relationship between the two processes. Cessation of carotenoids synthesis at water potentials higher than the minimum required for growth, probably suggests that the cessation is due to the effect on growth. Unlike growth, carotenoids synthesis in *R. glutinis* is stimulated by marginally low water potentials. In *Sacchromyces cerevisiae* similar stimulation of biosynthetic processes relatively to cell growth at low water potentials has been reported (Brown, 1976). Negative effects on both processes at lower water potentials could be due to the diversion of energy for the increased accumulation of solutes in response to water stress. Brown (1976) speculated that progressive diversion of energy into glycerol [solute] production eventually reaches a proportion sufficient to starve other biosynthetic activities. Carotenoids have not been assigned an important role in yeast cell physiology. Its supposed role as a photoreceptor may not be important except in photosynthesizing algae. Thus biosynthesis of carotenoids is certain to be placed low in a scale of priorities, and jettisoned early when subject to stress. In addition to such indirect effects on carotenoids biosynthesis via growth, its distinctly possible that low water potentials and solutes directly affect numerous enzymes that participate in the biosynthetic process. Such effects may account for the relatively higher level of inhibition in sucrose amended medium. Effect of sucrose on the enzyme isocitrate dehydrogenase in the xerotolerant yeast *Saccharomyces rouxii* has been reported (Brown, 1978). Though there are metabolic differences between xerotolerant yeasts and their non-tolerant counterparts, there is no evidence that the

enzymes associated with these differences have peculiar water relations (Brown, 1978). Thus reduced water potential and high solute concentrations could affect growth and carotenoids biosynthesis through their direct effects and through enzyme inhibition.

The synthesis of torularhodin seems to be particularly affected by the low water potentials. At very low water potentials, general depression in carotenoids biosynthesis may reduce the starting material necessary for the formation of torularhodin. The decline of the torularhodin content (as a percentage) at moderately low water potentials requires a different explanation. An incomplete biosynthetic process could be most likely. The conversion of torulene to torularhodin is the last step in the process. Significant quantities of γ-carotene needs to be synthesized for this conversion to take place. The colour of the cultures at these water potentials, as well as the absorption spectrum indicate that appreciable quantities of γ-carotene are synthesized. However, the conversion of γ-carotene to torulene requires atmospheric oxygen, which is incorporated to the torulene molecule (Simpson, 1972). Under low water potentials, respiration has been reported to increase in order to meet the increased energy demands for the solute accumulation and maintainance of growth (Wilson and Griffin, 1975). Due to the already fully exploited respiratory capacity, oxygen requirements for the synthesis of the torulene molecule are not likely to be met, thus lowering the torulene and torularhodin contents. General inhibitory effect on enzymes may also limit the final step of the biosynthetic process. These results suggest that, in response to moderately low water potentials, the carotenoids biosynthetic process undergoes qualitative adjustments. Severe stress however, seems to cause complete cessation of the process.

The carotenoids biosynthesis in *R. glutinis* is relatively unaffected by light. The results suggest that initial exposure to light is critical for the process to begin. Once its triggered, the process of biosynthesis is insensitive to light. Nakayama *et al.*, (1954) reported that light has a simple quantitative effect on carotenoids biosynthesis in *R. rubra* and *R. peneus*. In *R. gracilis* the effect has been qualitative (Simpson *et al.*, 1964). The response of *R. glutinis* seems to be similar to that of *R. rubra*. This quantitative effect of light in *R. glutinis* probably suggests a differential response to light by the different species of *Rhodotorula*. This could be satisfactorily explained on the basis of differences in the requirements for the expression of genes responsible for enzyme activity. In a number of mycelial fungi, a short exposure to light and oxygen is essential for significant pigmentation to occur (Simpson, 1972).

Results on the effects of age of culture on carotenogenesis are consistent with the general model of carotenoids biosynthesis proposed by Goodwin (1971). According to his model, carotenoids biosynthesis in yeasts occurs in three distinct phases; a) an initial lag phase during which growth takes place, b) an increasing phase of carotenoids biosynthesis and c) a declining phase during which the carotenoids disappear. Results presented here agree with such a scheme. The period from the third to the fifth day probably constitutes the increasing phase during which a remarkable increase in the carotenoids content was observed. From the fifth day, the process seems to stabilize. Comparatively high torularhodin content during these phases provides further evidence that the process is carried out to the completion. The increase in the torularhodin content after the fourteenth day indicates that the carotenes are increasingly converted to xanthophylls during the final phase.

The carotenoids produced by the yeast *R. glutinis* undergo qualitative and quantitative changes in response to environmental conditions. This offers potentials in industrial exploitation of this yeast for the purposes expounded in this thesis. However, industrial applicability lies in its synthesis of β-carotene under specified environmental conditions. Presently available methods of carotenoids separation need to be improved upon to enable such a study.

Chapter 6

Separation of biological chemical compounds

6.1. Introduction

The constituents of most biological substrates are complex mixtures of groups of closely related chemical compounds (monosaccharides, amino acids, polysaccharides, and proteins) and their derivatives. Analytical determination of these substances requires complete separation of individual components. Methods of separation that could potentially be employed in the analysis of biological chemical compounds have been employed in other areas of chemistry from the ancient times. Simple separation methods such as phase separation by filtration (removal of a solid phase) and drying (removal of a liquid phase) have been routinely employed in numerous chemical analyses. Phase separation into immiscible solvents is still a widely used method of separation of solutes with different solubility coefficients. Crystallization and distillation are other methods traditionally employed in chemical separations.

The separation capacity of the above methods (filtration, drying, crystallization, distillation) is limited to those compounds which are not closely related chemically, and occurring as major components in a mixture. They are in general used for the elimination of minor amounts of impurities. These methods are less successful in the separation of minor components and chemically related compounds such as biological compounds. Further, characteristics such as the high temperatures they employ to achieve separations precludes application of these techniques in the separation of compounds which are labile under such conditions. These methods are also difficult to adapt to automation. The complete separation of biological compounds is complicated by their highly complex structures, occurrence in small amounts, and extremely labile nature. These characteristics demand adoption of methods that are capable of achieving the separations while preventing the loss or modification of compounds during the separation.

This has provided the impetus for the development of methods that avoid extremes of pH, temperature and salt concentrations and suitable for the separation of substances in

solution. The distinguishing features of these methods are their sophistication and sensitivity (to the picogram level) and, in particular, the mild experimental conditions they employ. These methods which include centrifugation, electrophoresis and chromatography are more sophisticated in that they exploit differences in chemical properties such as polarity and molecular size and structure to achieve separations. The chemical characteristics of the compounds these methods exploit in separations and a high degree of automation have improved the accuracy and reproducibility of data generated by these methods. The technique of chromatography will be considered in detail here.

6.2. Chromatography

Chromatography as a method of chemical separation has been used since ancient times and it is by far the most widely used separation method at present in biochemical studies. The resurgence of the use of this method was prompted by its successful application in the separation of plant pigments (Tswet, 190). After another lapse of application for more than 25 years, its widespread use resumed in the 1930's when Kuhn and Lederer (1931) repeated some of Tswet's experiments. At present, extremely sensitive and highly automated chromatographic systems that achieve separation by employing different chemical principles are being used in the analysis of numerous biochemical compounds.

In chromatographic separation methods separation results from the differential distribution of the components of the mixture between a two-phase system: the stationary phase which may be solid, liquid, or solid/liquid mixture which is immobilized, and the mobile phase which may be liquid or gas. Practically, this is achieved by adding the mixture of solutes to be separated to the mobile phase, which is made to flow continuously at a constant rate past the stationary phase. The components which exhibit a greater affinity for the stationary phase are retained within the system for a longer time than those components which favour the mobile phase. The differential migration results from the differential distribution of the various components between the mobile and stationary phases. Exchange of the solutes between mobile and stationary phases takes place during the operation. The separation finally obtained depends on the relative distribution of the solutes between the two phases and on the number of exchanges during the passage of the solutes over the stationary phase. A wide variety of chemical and physical interactions which molecules of chemical substances can undergo with the stationary phase are used for chromatographic separations. These include polar forces arising from permanent or induced electric fields associated with both solute and solvent molecules, or Van der Walls

forces which depend on the relative masses of the solute and solvent molecules. The basis of chromatography depends on exploiting different intermolecular forces to achieve separation. Chromatographic methods are classified into three categories on the basis of the predominant types of reactions that cause interchange between the two phases: (a) adsorption chromatography (b) partition chromatography, and (c) ion-exchange chromatography.

6.2.1. Adsorption chromatography

Adsorption chromatography is the earliest developed method of chromatographic separation which in recent years has been virtually replaced by modern methods such as partition, ion-exchange and paper chromatography. In this method, separation of components depends upon differences in their degree of adsorption by the adsorbent and solubility in the solvent used for separation. Attraction of molecules to the adsorbent does not involve electrostatic forces, but adsorption. The nature of the separation process requires that the adsorbent is insoluble in the solvent system used. Since only the surface of the adsorbent particle is accessible to the solute molecule, reduction in particle size of the stationary phase increases the surface area and hence the number of active sites per unit weight. This effectively increases the adsorption capacity of the adsorbent. Polar or nonpolar adsorbents can be used depending on the nature of separation required.

6.2.1.1. Thin Layer Chromatography

Thin Layer Chromatography (TLC) is one of the widely used methods of chromatography that exploits the adsorption phenomenon. In TLC, a glass sheet (20 x 20 cm) is coated with a slurry of adsorbent in water and then dried at 100-120°C for approximately 1-2 h. The normal thickness of slurry layer used is 0.25 mm for qualitative analysis, though layers up to 5 mm thick may be used in preparative analyses. The sample is applied to the plate by means of a micropipette as a spot, about 2.5 cm from the end. The solvent is evaporated from the sample by the use of an air blower. Separation takes place in a glass tank which contains the developing solvent to a depth of about 1.5 cm in a solvent saturated atmosphere. The thin layer plate bearing the sample is placed vertically in the tank and separation of the compounds then occurs as the solvent travels up the plate. The system is maintained at a constant temperature. The distance moved by a solute in the direction of solvent flow during a chromatographic separation is characterized by the term R_f value, which is defined as:

$$R_f = \frac{\textit{Distance travelled by solute}}{\textit{Distance travelled by solvent}}$$

This value is a constant for a particular compound under standard conditions, and reflects the distribution coefficient for that compound. Adsorbents usually contain a fluorescent dye so that the separated compounds appear as blue or green areas when a plate is examined under ultraviolet light after development. Spraying the plate with 50% sulphuric acid or heating is done to trace separated compounds. These spots are scraped off the plate, eluted with a suitable solvent, the adsorbent removed by centrifugation, and quantified by spectrophotometric techniques or further chromatography. Thin layer chromatography is a versatile technique which offers unlimited choice of adsorbents and quick separations due to the compact nature of the adsorbent layer. The first effective form of TLC was used by Maclean and Hall (1949). Stahl (1956) introduced new efficient spreading technique of the stationary phase, which increased the popularity of this method.

Table 6-1 presents the diverse adsobents and solvent systems reported in literature for the analysis of carotenoids extracted from numerous sources. Usually TLC is sensitive to microgram quantities of a compound to be separated. Two dimensional separation is employed in the resolution of certain separations. However, its major disadvantage is the unsatisfactory reproducibility of the R_f values.

6.2.2. Partition chromatography

Partition systems, in their simplest form involve two solvents which are immiscible. However, many recently developed media consist of an aqueous phase immobilized on a supporting medium (silica gel) and an immiscible organic mobile phase. Selective retardation of solutes in the column is primarily caused by their varying distribution between the mobile organic phase and the stationary aqueous phase, though adsorption is also involved. The introduction of cellulose in the form of filter paper as a supporting phase enabled separations in the microgram scale and permitted the introduction of the two dimensional separation principle. Most of the supporting phases in common use in partition chromatography are hydrophilic and preferentially retain the more polar of an immiscible solvent pair, usually the aqueous phase. Gas-Liquid Chromatography (GLC) is a commonly used mode of chromatography that employs partition phenomenon.

Stationary phase	Mobile phase	Reference
Silica gel and Ca $(OH)_2$	P.E[1]. and Benzene (1:1)	Winterstein *et al.*, (1960)
Silica gel and Ca $(OH)_2$	P. E. and Benzene (2:3)	Isler *et al.*, (1961)
Silica gel G	P.E., Benzene, Alcohol (50 : 50:1) to (100: 20: 7)	Grob *et al.*, (1961)
$Al_2 O_3$	P.E., Benzene and Alcohol (100 : 100 : 1)	Grob *et al.*, (1961)
Kieselguhr and vegetable oil	Methanol, Acetone and Water (20 : 4: 3)	Egger, (1962)
Silica gel G	P.E., Benzene and Acetone (160 : 40: 4)	Rispoli and Di Giacomo, (1963)
Cellulose	P.E., Benzene, Chloroform, Acetone and 2-propanol	Sherma, (1970)
Silica and $Ca_2 CO_3$	P.E., Acetone and 2-propanol	Bjornland and Aguilar-Martinz, (1976)

1= Petroleum ether

Table 6-1: Adsorbents and solvent systems employed in the TLC analysis of carotenoids

6.2.2.1. Gas-Liquid Chromatography

This method employs an inert gas (N, He, Ar) as the mobile phase in a partition column which is at a constant, elevated temperature. The stationary phase is usually a high boiling point liquid supported on a relatively inert porous material. The solute mixture is introduced to the column in liquid, solid or gaseous form and transported through it in the vapour state. This need for vapourisation limits its usage only to those compounds that are stable at high temperatures. Carotenoids cannot be separated by GLC without prior perhydrogenation (saturation), a procedure which may give rise to artefact formation. Resolution of the mixture occurs as a result of their varying distribution ratios between the gaseous and the liquid phases. The use of this method is restricted to those compounds which are volatile without decomposition at temperatures up to 250 °C. Its most valuable application has been in the analysis of volatile substances, particularly the aroma components of foods. Gas-Liquid Chromatography is a major advancement over TLC in resolution, reproducibility and automation. Its major disadvantage however, is its inability to separate numerous non-volatile, polar and thermally labile compounds that are preponderant in nature.

6.2.3. Ion exchange Chromatography

This method is applicable to charged solutes which can be separated on the basis of their tenacity of binding to oppositely charged chemical groups presented on the stationary medium. More efficient separations are usually achieved when the stationary phase is of low ionic strength. Ion exchange materials are highly insoluble acids or bases. In general an ion exchange column may contain an exchanging anion (e. g. quaternary amonium group) or a cation (e. g. sulphonic acid group). The ion exchange resins are only partially characterized by their functional groups. The nature and the number of exchanging groups in the resin as well as the chemical properties of the resin matrix, to which the groups are attached, can be varied. Ion exchange resin used for characteristic purposes should be monofunctional with respect to the functional group. Recently developed synthetic resins with high exchange capacities are commercially available. The flow rates used in this method are often considerably greater than those used in other methods.

6.2.4. Reversed phase chromatography

The surface of a silica particle used as the stationary phase in any of the above methods of chromatography is covered with slightly acidic silanol groups which react with polar solutes that come into contact with it. The polar components of a mixture are retained on the stationary phase (Si) by these interactions as in adsorption chromatography. In reversed phase HPLC, the characteristics of the silanol groups on the surface of the silica particles (the stationary medium) are reversed from polar to nonpolar by chemically reacting with compounds such as octadecyl trichlorosilane, C_8 or C_{18}. However, some active silanol groups left behind due to the incomplete reactions with these compounds carry out separations as a result of adsorption and partition mechanisms. The substituted surface after hydrolysis bears nonpolar characteristics. This hydrocarbon layer chemically bonded to the silica, unlike polar silanol groups, interacts with nonpolar components of a mixture. The introduction of reversed phase stationary phases have enabled the application of chromatography in the separation of nonplar compounds which abound in biological systems.

6.3. High performance liquid chromatography (HPLC)

The development of GLC reduced interest in liquid chromatography until recently, when HPLC almost began superseding GLC for many analytical purposes. High Performance Liquid Chromatography, while eliminating the need for vapourising the sample, rivals GLC with high resolution, speed, sensitivity, automatic operation and the unsurpassed range of applications. High performance liquid chromatography is now recognized as a powerful analytical and preparative technique. The principle of operation of HPLC is that is common to liquid chromatography; it exploits different affinities of the components for a stationary phase packed into a column and a mobile liquid phase that percolates through. While the use of a single solvent (isocratic separation) may often be adequate to achieve a desired separation, more complex mixtures may require a gradient elution. In gradient elutions the eluting power of a mobile phase is increased with the gradual addition of a more polar solvent. Separation is determined by monitoring the column effluent using a sample detector. A chromatogram relates the concentration of the components in the mobile phase with time of retention by the column. Efficient separations require minute and regular-shaped support media, a constant flow rate of the mobile phase and an efficient detection system. This method is often referred to as high pressure liquid chromatography, a name descriptive of the comparatively high pressures it employs (40 MPa). However, the

nomenclature high performance liquid chromatography better describes all aspects of this method relating to speed, capacity and sensitivity.

6.3.1. HPLC equipment and operation

An HPLC system consists of five separate components: the mobile phase delivery system (pump), the injector, the column, the detector and the recorder. While the column is the component that effectuates the separation, the other components of the system provide support necessary for efficient and reproducibile separations. Major manufacturers usually build individual, compatible modules that are linked together.

Pumps: Chromatographic separation requires the delivery of the mobile phase through the stationary phase at a predetermined constant rate. In modern HPLC systems this is mechanically achieved with the use of pumps. The pump is usually the major component of the solvent delivery system, which incorporates facilities for degassing the solvents and mixing them in solvent gradients. Efficient and reproducible separations require a constant flow rate of solvent through the column. Pulsation within the system can result in a noisy detector baseline and thus raise the detection limits in sensitive assays. Mechanical reciprocating pumps (single, dual or triple-headed) with electrically driven pistons are usually employed. A pump that achieves virtually pulse free flow rates with the use of electronic pulse compensation circuitry has recently been introduced by a major supplier of HPLC equipment.

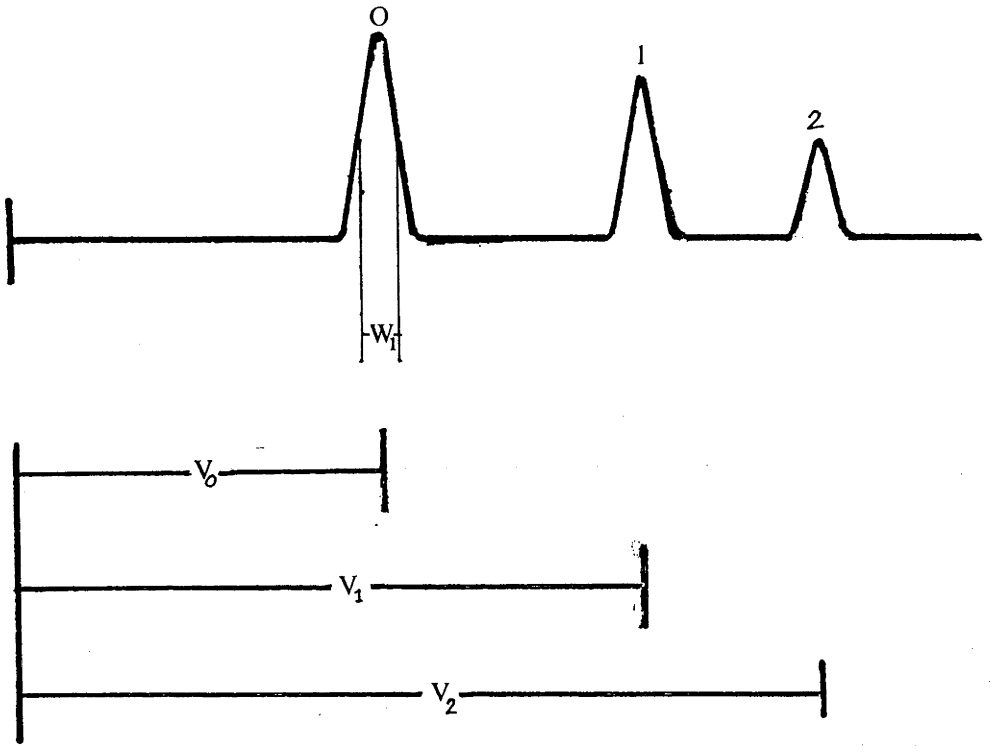
Injectors: For accurate separations, the sample needs to be injected at the top of the column without any dilution. Syringes and valves are the two main injection devices available for this purpose at present. Syringes inject the samples in-line at the top of the columns. Valves inject samples into loops which subsequently are brought in-line with the HPLC system. Injection valves provide major advantages of precision, reliability, and ease of operation and are therefore, the widely used system in modern HPLC apparatus.

Columns: The column is the single most important component of the HPLC assembly which determines, together with the mobile phase, the quality of a separation achieved. The narrow metal tube containing the tightly packed stationary phase constitutes the column. Microfilters are usually placed at column ends to prevent particulate matter accumulating on top of the stationary phase material. At present the majority of the packing materials for HPLC columns are based on silica gels due to its versatility as an adsorbent. These gels consist of fused aggregates of colloidal silica which have surface

areas up to 800 m^2 and pore sizes of 5 x 10^{-6} mm Adsorbents with high surface area, although producing high resolution, have a restricted polarity range and can separate only solutes that have polarities close to that of the mobile phase (Scott, 1976). However, microparticulate supports of 5-10 μm diameter, which render separations extremely efficient, are being widely used. A large number of different types of modifier bonded silica packed columns are presently used in different modes of chromatography. Octadecanoylsilyl (ODS), amino (NH_2), and cyano (CN) groups are widely used in reversed phase HPLC columns. Normally, a small precolumn (40 mm long, 3 mm internal diameter) is used in order to filter particles in the sample and solvents that may accumulate on the top of the column. The precolumn is packed with microparticulate material similar to those in the analytical column. The relatively high pressures (41.3 MPa) employed in HPLC systems have led to the replacement of glass tubes with stainless steel column housings. A short column together with a narrow diameter column packing is necessary to achieve rapid, high efficiency resolutions. Most analytical columns are 10-20 cm in lengths and 4-6 mm in internal diameter. The length to diameter ratio should be at least 10 and preferably larger. An important recent advancement in column technology is the emergence of radially packed columns.

Characterization of a new column in terms of a number of theoretical criteria prior to use is essential to determine its efficiency, and to be able to compare its performance over time. Two of the most important of these criteria are the capacity factor (k') and the number of theoretical plates (N). The measurement of the capacity factor ensures that the sample has been sufficiently retained on the column for separation to occur. The capacity factor and the number of theoretical plates are calculated as shown in figure 6-1.

Detectors: The concentrations of individual components of a mixture separated by the HPLC column and being eluted are monitored by a detector. Efficient use of a HPLC column depends on the use of a detector of high sensitivity. Detection can be based on the measurement of a bulk property of the eluting solvent (refractive index) or a physical property of the solute. The most widely used solute property is ultraviolet light (uv) absorption. The variable wavelength uv detectors are being increasingly used.



Capacity factor k' = $\frac{V_1 - V_2}{V_0}$

Separation factor α = $\frac{V_2 - V_0}{V_1 - V_0}$

Number of theoretical plates N = $16\left(\frac{V_1}{W_1}\right)^2$

V_o, V_1, and V_2 are ml equialents of the retention times of the peaks 1, 2 and 3 respectively

Figure 6-1: Calculation of HPLC column parameters

6.4. Carotenoids

Carotenoids are bright yellow to red pigments with a widespread distribution in all photosynthetic and in many nonphotosynthetic organisms. They are synthesized *de novo* by higher plants, algae, fungi and bacteria. They are lipid soluble pigments located in the cytoplasm of plant cells, and could be extracted with benzene, ether, CS_2, ethanol or chloroform. Carotenoids is a term used to include carotenes (hydrocarbons) and xanthophylls (their oxygenated derivatives). The oxygen function in xanthophylls can take the form of a hydroxyl, epoxy, or a carboxyl group. The vast majority of the more than 400 naturally occurring, identified carotenoids are tetraterpenoids with a highly branched C_{40} skeleton corresponding to eight isoprene units which reflects their mode of biosynthesis. Carotenoids are formed by the joining, tail to tail, of two units each consisting of four isoprenoid units joined head to tail, thus:

ipipipippipipipi

All naturally occurring carotenoids represent variations on this basic theme. The arrangement of the isoprene units at the center of the molecule is characteristically reversed. Another distinguishing feature of the carotenoids is the array of conjugated double bonds which are responsible for light absorption. The chain of conjugated double bonds is mainly or entirely the chromophore. Modification of the acyclic skeleton by hydrogenation or dehydrogenation leading to cis-trans isomerization, cyclization, and oxygenation yield the great variety of carotenoids. Carotenoid classification is based on the nature of their carbon (C_9) end groups which are given the prefixes

acyclic (ψ)

cyclohexene (β)

cyclohexene (ε)

The specific designation for the carbon end group is prefixed to the semisystematic stem name 'carotene'. The nomenclature of carotenoids with modification of the carotene skeleton, changes in hydrogenation level, configuration and oxygen function in xanthophylls, has been established (Commission on biochemical nomenclature, 1971). The major carotenoids that occur widely in nature are β-carotene, lutein, violaxanthin and neoxanthin. Those occurring in smaller amounts are α-carotene, β-cryptoxanthin and zeaxanthin. A basic pathway for the biosynthesis of carotenoids in plants and microorganisms has been suggested (Figure 6-2). According to this pathway, the acetate

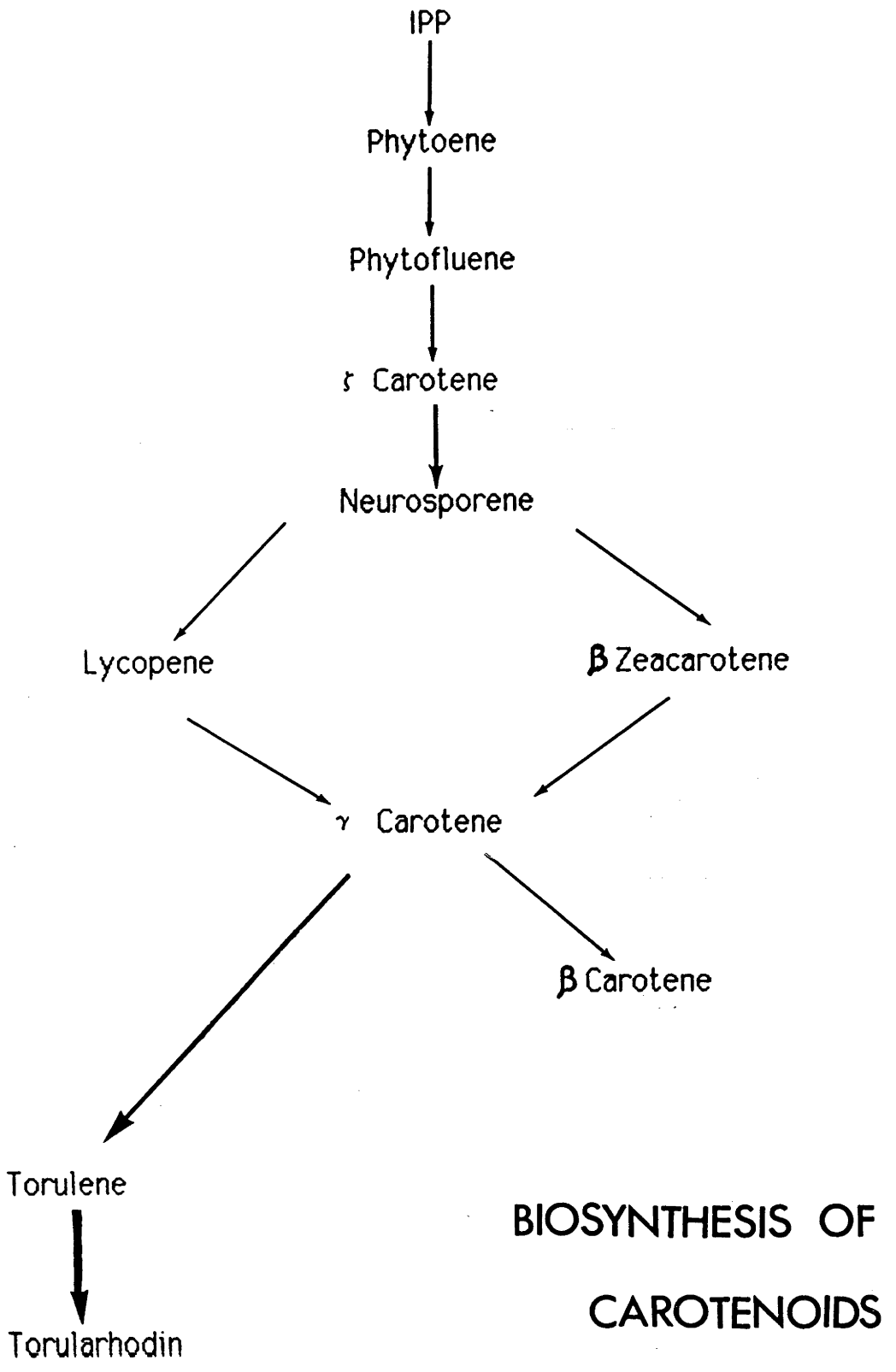


Figure 6.2 Schematic pathway of the biosynthesis of carotenoids (adated from Simpson, 1972)

molecule is the original building block of all isoprenoids. This is converted to mevalonic acid (MVA), which in turn is converted to isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMPP). Isoprenoid precursors are formed by the addition of five carbon units to DMPP. Head-to-head condensation of C_{20} units yields phytoene, the first acyclic carotenoid to be synthesized. Phytoene is converted to lycopene and the synthesis continues with cyclization to form β- and γ- carotene.

6.4.1. Distribution of carotenoids in fungi

Carotenoids have been detected in some 200 fungal species. Approximately 100 fungal species do not synthesize detectable amounts of carotenoids. The possible use of carotenoids as taxonomic markers of fungi, in addition to other biochemical characteristics was suggested by Valadon (1976). Simple carotenes, especially β-carotene and γ-carotene, are the most frequently occurring carotenoids in fungi. Quantitatively, β-carotene is often the major pigment. Carotenoids in lower fungi (Chytridiomycetes and Zygomycotina) consists predominantly of carotenes with few xanthophylls reported. In Ascomycetes, carotenes predominate quantitatively, with xanthophylls occurring with some frequency. In Basidiomycotina and deuteromycetes, xanthophylls also have been found in approximately 50% of all species in which carotenes have been found, but carotenes are usually predominant (Goodwin 1973, 1976) . The characteristic fungal xanthophylls are carboxylic acids which involve the oxidation of one of the sorbenmethyl groups as in torularhodin. Such xanthophylls as torulene and torularhodin are characteristic of red yeasts.

6.4.2. Chromatography of carotenoids

Chromatography is the most useful method for the purification of carotenoids. The method of chromatography, nature of the adsorbants and the solvent system to be chosen depend on the particular carotenoids to be separated. Procedures involved in the chromatography of carotenoids have been described by Britton and Goodwin (1971) and Davis (1976). Chromatography of carotenoids generally consist of preliminary separation by adsorption chromatography on a column of alumina and separation into individual components by TLC in two or three subsequent combinations of stationary and mobile phases. Preliminary column chromatography separates the carotenoids mixture into compounds of similar polarity (carotenes, monohydroxy compounds and dihydroxy compounds). In such adsorptive chromatographic systems the carotenes move with the solvent front. Rate of movement (R_f value) decreases with increasing number of hydroxy or epoxy groups.

6.4.2.1. Thin Layer Chromatography of carotenoids

The groups of compounds of similar polarity to which, the carotenoid mixture is separated by preliminary chromatography contain several individual carotenoids with many impurities. Thin Layer Chromatography, which is a more selective technique, separates compounds of similar polarity. This is usually done in two stages. The first stage involves separation on silica gel or alumina, with the solvent used for elution from the column. The carotenoid samples obtained from the first TLC on silica gel are further purified by TLC on MgO or $Ca(OH)_2$ with acetone-light petroleum or benzene-light petroleum mixture as the solvents. Separation on the second adsorbent depends mainly on the number and nature of double bonds in the carotenoid molecule. After TLC separation on MgO, the carotenoids are finally purified by TLC on silica gel. During TLC, carotenoids are particularly vulnerable to isomerization or decomposition by acid, light, oxygen and heat. Precautions taken to minimize the danger of such modifications include avoiding the use of acidic or strongly alkaline adsorbants and solvents. Application of the sample and development should be done under subdued light or darkness. In the case of extremely oxidative samples, maintaining a nitrogen atmosphere in the chromatography tank becomes necessary. The coloured carotenoids can be easily located on the developed TLC plate. Locating colourless compounds, however, requires examination of the plate under ultraviolet light. Compounds are scraped and eluted from silica gel or alumina with diethyl ether, or acetone in the case of MgO.

6.4.2.2. HPLC of carotenoids

Column and thin layer chromatographic systems traditionally employed in the separation of carotenoids have several limitations. In these methods, separation of the whole range of pigments with good resolution in a single analysis is impossible. This necessitates preliminary separations such as phase separation, successive thin layer chromatography on different adsorbent-solvent combinations, or two-dimensional thin layer chromatography. Such excessive manipulation of chemically fragile carotenoids often leads to coloured and uncoloured artefact formation. After separating torulene on cellulose thin layers to two red and orange spots, Simpson *et al.* (1964) concluded that torulene had undergone isomerisation. The carotenoids as a class of compounds lend themselves easily to such structural alterations. The use of silica gel and other polar stationary phases aggravates this source of error. Further, reliable quantification by TLC and paper chromatography require comparatively larger amounts of the separated pigments, beyond the levels at which minor pigments occur in nature. Recovery of visible parts of the chromatogram by

scraping and subsequent elution is often the other major source of error in TLC. Overlooking minor pigments, errors in recovering spots with unsharp or overlapping zone boundaries often limit recovery to 90-95% even when extensive precautionary measures are taken.

Despite the original application of chromatography for the separation of carotenoids (Tswet, 1901), widescale application of the HPLC in the analysis of carotenoids earnestly began only in 1971 (Stewart and Wheaton, 1971). These workers employed HPLC for the separation of carotenoids from orange peel. Rather long retention times (4-5 h), by the standards achievable with HPLC, obtained in this study can be attributed to the particle sizes of the stationary phase material used. Reviews of literature on the use of HPLC for carotenoid analyses have been published (Taylor and Ikawa, 1980; Schwartz and von Elbe, 1982; Taylor, 1983; Ruddat and Will, 1985). The high resolution capabilities of HPLC columns make it possible to separate the complex mixtures of closely related carotenoids. Analysis times including sample preparation are significantly less than in other chromatographic methods. Saponified extracts can be directly injected in to the column unlike traditional chromatography in which preliminary column chromatography and three subsequent steps of TLC are involved. Early methods developed for the separation of carotenoids by HPLC utilized adsorption chromatography with nonpolar mobile phases. Since then, a variety of column packings and mobile phases have been used in the HPLC analysis of carotenoids. Table 6-2 presents a summary of columns, solvent systems and other parameters that have been used in the HPLC analysis of carotenoids. The use of micro particles (5-10 μm) of porous silica and reverse-phase stationary phases have been reported. Solvents used are n-hexane, petroleum ether with a polar solvent, or a mixture of water and acetonitrile in the reverse-phase. Quantitative limits of analysis are usually in the low nanogram range and detection is normally carried out at 450 nm. The column is flushed with solvent for 15-30 min prior to use and an aliquot of the carotenoid mixture (10-45 μl) (dissolved in the initial composition of the mobile phase) is injected. The appropriate gradient elution sequence is started and the elution is carried out at a predetermined flow rate, usually 1-2 ml/min. Degassing of solvents is an extremely important practical consideration that increases the reproducibility of the separation and the column lifespan.

Column	Solvents	Flow rate	R.T.[i] for β-car.	Reference
Spherisorb 5 μm	0-40% acetone in hexane containing 0.1% methanol	3 ml/min	3 min	Fiksdahl et al., (1978)
μ porasil 30 Lichrosorb RP8 μ bondapak C18 Partisil PXS10 Partisil PXS5	Isoctane, Chloroform, A[2]. Diethyl Ether Methanol 8% chloroform, A.	2 ml/min	12.5 min	Zakaria and Simpson, (1979)
Lichrosorb RP18	Methanol, A., Water(25:75)	1.5 ml/min		Braumann and Grimme, (1981)
Ultrasphere ODS 5 m	2-propanol, A., Water 30:70-55:45	40 ml/h	36 min	Will and Ruddat, (1984)

[i] = Retention time, [2]= Acetonitrile

Table 6-2: Columns and solvent systems employed in the HPLC analysis of carotenoids

Chapter 7

rp-HPLC analysis of variations in β-carotene biosynthesis in *Rhodotorula glutinis* in response to cultural conditions

7.1. Introduction

The hypothesis presented in this dissertation regarding the role of the yeast *Rhodotorula glutinis* in tea flavour synthesis (subsection 1.3.1) rests on several presumptions. The most important of these is that the carotenoids (β-carotene) biosynthesis by the yeast *R. glutinis* undergoes seasonal variations, presumably in response to environmental conditions. Proof of this hypothesis is a prerequisite for an explanation of seasonality of tea flavour development on the basis of yeast activity. An investigation into variations in carotenoids biosynthesis by *R. glutinis* revealed that the process of biosynthesis of the total carotenoids complex is governed by variations in environmental conditions (Chapter 4). The carotenoids complex of the yeasts however, is a mixture of β-carotene, γ-carotene, torulene and torularhodin. The proposed role of 'carotenoids' in tea flavour development is expected to be solely due to β-carotene.

Study of the variations in β-carotene biosynthesis requires accurate analysis of the yeast carotenoids complex. Phase separation, column chromatography and thin layer chromatography (TLC) have been employed individually or in different combinations in separations of yeast carotenoids. Deficiencies of these traditional methods have been pointed out in the preceding chapter. Exposure to atmosphere and incomplete recovery from the thin layer plate are series defects which are conducive to artefact formation and inaccurate quantitative estimation of the carotenoids. In the earliest reported separation of the yeast carotenoids, Nakayama *et al.* (1953) used column chromatography with a 2:1 mixture of MgO and Hyflo Supercel as the stationary phase. Peterson *et al.* (1957) separated torularhodin by liquid-liquid phase separation prior to column chromatography on a similar stationary phase with hexane and absolute ethanol as solvents. In this study, β- and γ-carotenes eluted together while torulene remained adsorbed to the stationary phase, necessitating subsequent recovery and quantification. The procedure described by Simpson *et al.* (1964) epitomises the laboriousness of, and the hazards involved in, the methods that

were available at the time. In this detailed study, the pigment complex was initially subjected to column chromatography, saponified, and separated in eight subsequent steps of TLC. Cellulose, MgO-Supercell, alumina grade i and alumina grade ii stationary phases with different combinations of petroleum ether, acetone and ethyl ether as solvents were used. After separating torulene on cellulose thin layers to two spots (red and orange), these workers concluded that the two pigments were isomers of torulene formed during the extraction and purification of carotenoids. Under such extensive handling as done in this experiment, the structural transformation of carotenoids becomes inevitable.

Reversed phase high performance liquid chromatography (rp-HPLC) is an analytical technique specially suited for the analysis of carotenoids. In this technique, the separation mechanism relies on the hydrophobic interaction between the nonpolar hydrocarbonaceous stationary phase, solute and mobile phase. Weak hydrophobic forces that perform separations are less likely to cause artefact formation than ionic and polar forces operational in adsorption chromatography. Oxidation of the sample is prevented by the exclusion of oxygen from the separation system. Degassing the solvents, which are the only carrier for oxygen, prior to use eliminates this problem. The saponified extracts can be applied directly to the HPLC column and the carotenoids are eluted from the column as pure compounds in small volumes. This provides a rapid, more sensitive and accurate method for the analysis of carotenoids. Application of the HPLC in the analysis of carotenoids from numerous substrates have been reported in literature. Zakaria and Simpson (1979) used HPLC to analyse carotenoids from tomato. Carotenoids of green leaves of various crop species have been analysed with HPLC by Braumann and Grimme (1981), Prenzel and Lichtenthaler (1982) and Takeo (1985). However, the reports of HPLC analyses of microbial carotenoids are scarce. Wright and Shearer (1984) reported the use of HPLC in the analysis of carotenoids of the marine phytoplanktons. A major boost to this area of research has been provided by the application of rp-HPLC in the characterization of carotene accumulation in *Ustilago violacea* (Will *et al.*, 1984; Will and Ruddat, 1985) using columns with different bonded phase silica stationary phases and numerous solvent systems. A review of the subject of the HPLC analysis of carotenoids has also been undertaken by the same authors (Ruddat and Will, 1985).

Despite lack of precedence, the separation of yeast carotenoids with HPLC should be a theoretical possibility mainly due to the chemical structures of the yeast carotenoids which are similar to those carotenoids that have been separated with HPLC. However, HPLC separations of the major xanthophylls occurring in red yeasts (torulene and torularhodin)

have not been reported. The only chemical pecularity of torulene and torularhodin that could be of consequence in a chromatographic separation is their higher polarity than the carotenes contained in the mixture. However, with the availability of stationary phases of varying chemical characteristics and solvent systems to enable desired elutions, the problem of separation of such a mixture of chemical substances seems surmountable. In view of the rapidity of analyses afforded, HPLC becomes the method of choice for the evaluation of the effect of environmental conditions on pigment biosynthesis in the yeast. This technique will also enable more accurate quantification of compounds since the recovery is complete unlike in TLC. The existence of these techniques prompted a study of the carotenoids biosynthesis by the yeast *R. glutinis*.

The results of the TLC analyses of the effect of temperature on the biosynthesis of yeast carotenoids by Nakayama *et al.* (1954) and Simpson *et al.* (1964) lack aggreement. Reports of experiments on the effects of age of culture, nutrition and irradiance on the biosynthesis of yeast carotenoids are not available in literature.

7.2. Materials and methods

7.2.1. Culture of yeasts

For the HPLC analysis of yeast carotenoids: The yeast *R. glutinis* was cultured at 25°C for 5 d for a pilot analysis of the carotenoids content synthesized under standard conditions. An aliquot (0.1 ml) from a suspension of yeast cells obtained from a stock culture maintained at 5°C was used for the inoculation. The cell concentration in the solution was adjusted to 1 000 ml^{-2} with the help of a haemocytometer.

The effect of age of culture on β-carotene synthesis: The yeast was cultured on 25 plates according to the method described above and incubated at 25°C. Samples of five plates each were taken after 3 d, 5 d, 7 d and 10 d incubation and carotenoids extracted for HPLC analysis. The experiment was repeated three times.

The effect of temperature of incubation on β-carotene synthesis: Five culture plates each were inoculated with the yeast and incubated at 5°C, 15°C or 25°C for 5 d. Carotenoids were extracted from the cells grown at each temperature and analysed by HPLC. The experiment was repeated three times.

The effect of C:N ratio on the β-carotene synthesis: Yeasts for this experiment were

cultured on the basal medium, the composition of which is given in appendix ii. The carbon to nitrogen ratio of the basal medium was altered by adding different proportions of glucose, yeast extract or both these compounds. The quantities added were as follows:

a). Basal medium + 2% glucose

b). Basal medium + 5% glucose

c). Basal medium + 2% yeast extract

d). Basal medium + 5% yeast extract

e). Basal medium + 2% glucose + 2% yeast extract

f). Basal medium + 5% glucose + 5% yeast extract

In each case five cultures were grown at 25°C and the carotenoids extracted for HPLC analysis. A set of cultures grown on unamended basal medium was used as the control.

The effect of illumination on β-carotene biosynthesis: The yeasts were grown without illumination for the entire period of culture, under illumination for the entire period of culture, 6 h period of illumination followed by darkness, and a 6 h period of darkness followed by illumination. After incubating for 5 d at 25°C, the cells were harvested and carotenoids extracted for HPLC analysis. The experiment was repeated three times.

7.2.2. Carotenoids sample preparation for the HPLC analysis

Extraction of yeast carotenoids: Cells from the(1 g) solid agar surfaces were scraped with a spatula and the carotenoids sample preparation for the HPLC analysis was done as follows; yeast cells were taken in a mortar and ground vigorously with 30 ml portions of acetone. Three such extraction were necessary to extract all carotenoids leaving a white cell mass. The carotenoids solution in acetone was transferred to a 250 ml separating funnel to which 25 ml of n-hexane was added. Distilled water was then added until the two layers separated. The upper hexane layer containing the carotenoids was washed three times with distilled water to remove traces of acetone and collected in a 25 ml volumetric flask through anhydrous Na_2SO_4 and made to volume.

Saponification: The extract in hexane was later dried in a slow stream of nitrogen and re-dissolved in 25 ml of ethanol. The carotenoids solution in ethanol was saponified by

adding 10 ml of KOH (60% w/v), and allowing to stand under nitrogen at 5°C overnight. Carotenoids were taken up in equal volume of diethyl ether and a dilute NaCl solution until two layers were formed. Etheral extracts were washed with water until free from alkali. These extracts were dried under nitrogen and dissolved in the initial phase of the solvents for immediate analysis, or stored under nitrogen at 5°C until analysed.

Removal of sterols: Sterols were removed by refrigerating overnight at 0°C.

Aliquots for HPLC: Carotenoids were dissolved in the initial phase of solvents (10:90 of 2-propanol and acetonitrile-water (90:10)) and filtered through an 0.45 μm pore size Acrodisc filter (Gelman Sciences, Ann Arbor, Mi, USA) mounted on a syringe. Samples of 25 μl of the filtered solution was injected into the HPLC column with a micro-syringe.

7.2.3. HPLC of carotenoids

The HPLC system used consisted of a Spectra-Physics SP8700 solvent delivery system (Spectra-Physics corporation, Santa Clara, CA, USA), an Alltech 605RPC (C_{18}) column (Alltech Associates Inc., Deerfield, Illinois, USA), a Micromeritics 788 variable wavelength detector (Micromeritics, Norcross, Georgia, U.S.A), and a Waters model 730 data module recorder (Waters Associates, Milford, Massachusets, USA).

Solvents: The solvents used in the analysis, 2-propanol and acetonitrile, were of the HPLC grade purchased from Waters Associate, Sydney. Water used as a solvent was deionized, distilled water. All the solvents were filtered through a 0.45 μm filter connected to the end of the delivery lines and immersed in the solvent reservoires. All solvents were degassed with helium, using the facility incorporated into the solvent delivery system.

7.2.4. Calculation of the number of theoretical plates of the column

As explained in the introduction, characterization of the HPLC column in terms of the number of theoretical plates is vital for the evaluation of its efficiency. This imaginary statistic is also important in monitoring the changes in performance of the column with continued usage. Calculations of the number of theoretical plates of the column was done according to the instructions of the manufacturer. Analysis of a test mixture (RP MIX-D) using a 65:35 mixture of acetonitrile and water as solvents at a flow rate of 1 ml/min and detection at 254 nm was prescribed to measure the efficiency and selectivity of the reversed phase column. The column peaks of two of the components of the mixture,

toluene and ethyl benzene (with retention times 9.9 min and 13.6 min respectively) are used to measure column efficiency by using the formula:

$$N = 5.54 \times \left(\frac{t_r}{W_{1/2}} \right)^2$$

in which,

N = number of theoretical plates,

t_r = millimetre equivalent of the retention time in minutes (mm)

$W_{1/2}$ = peak width at 50% height (mm)

7.2.5. Determination of optimum chromatographic conditions for the separation of β-carotene

Since the objective of the study was rp-HPLC analysis of the yeast carotenoids for β-carotene, the solvent composition and gradient, and HPLC conditions (operational pressure, flow rate and wavelength for detection) that gave the optimum retention time and best resolution had to be determined. This was achieved by the chromatography of commercially purchased β-carotene (Sigma chemicals, St. Louis, Mo, USA). The chemical was dissolved in the initial phase of the solvent mixture to be used and chromatographed. Numerous isocratic and gradient systems were attempted. The column was flushed with solvents between runs until the pressure returned to the initial level and the recorder base line was stable.

Calibration of the detector: Stock solutions of synthetic β-carotene were prepared by weighing 1 mg into a 100 ml volumetric flask and bringing to volume with tetrahydrofuran (THF). Three working standards were made by taking 1, 2, and 3 ml from the stock solution. At each concentration, six replicates were analysed. The peak areas at different concentrations were plotted against concentration.

7.2.6. Identification and quantification of yeast carotenoids

Samples of saponified extracts of yeast pigments were subjected to HPLC under conditions described above. The peaks having retention times approximating to that of β-carotene were collected and their visible light absorption spectra were compared with that of β-carotene. Absorption at λ_{max} was used to calculate the concentration of β-carotene in solution using the formula given in Chapter 5.

7.3. Results

7.3.1. Calculation of the number of theoretical plates

Figure 7-1 shows the chromatograph of the analysis of the test mixture RP-D provided by the column manufacturer. Chromatogram of the analysis performed by the manufacturer is given in Appendix C. Retention times obtained were within 1-2% of the times reported in the analysis of the manufacturer. The components of the mixture uracil, phenol, benzaldehyde, n-n-diet-m-toluamide, toluene and ethyl benzene were retained for 2.04, 3.25, 4.43, 5.28, 9.9 and 13.62 min respectively. The peak for toluene was used for the calculation of the number of theoretical plates. According to the calculations based on a retention time of 9.9 min for toluene, and a peak width at half height of 3 mm, the column had a theoretical plate equialent of 26 000.

7.3.2. Optimisation of chromatographic conditions

Figures 7-2-7-6 show high pressure liquid chromatographs of commercially purchased β-carotene, with the solvent systems consisting of varying proportions of 2-propanol and aqueous (90%) acetonitrile. Retention time progressively decreased with careful increase in the 2-propanol content in the initial phase while keeping the flow rate constant. Even though elution of β-carotene could be achieved with a narrower gradient (starting with a higher prpoportion of 2-propanol), the need to achieve a satisfactory capacity factor for torularhodin (contained in the yeast pigments complex) necessitated starting the gradient with a lower proportion of 2-propanol. Due to its high polarity torularhodin could be expected to elute without being retained at high proportions of 2-propanol in the solvent mixture. Therefore, the analysis began with a 10:90 mixture of 2-propanol and 90% aqueous acetonitrile. The optimum retention time of 14 min for β-carotene was obtained with a gradient that reaches 45:55 in 40 min at a flow rate of 25 ml/h. The detector sensitivity was set at 0.1 I.U.F.S. and the recorder speed at 0.5 cm/min. Operational pressure was 10 MPa at the beginning of the gradient and gradually increased to around 20.6 MPa when the 2-propanol content increased in the gradient. Preliminary peaks a and b, which did not have absorption spectra of any known carotenoids were considered to be impurities. Quantitative recovery of β-carotene was examined by collecting the eluted pigment and spectroscopic determination of concentration. Isocratic elutions generally resulted in longer retention times.

Figure 7-1: Chromatogram of the test mixture RP-D (Peak numbers 1 = uracil, 2 = Phenol, 3 = Benzaldehyde, 4 = N-N-Diet-M-toluamide, 5 = toluene, 6 = benzene)

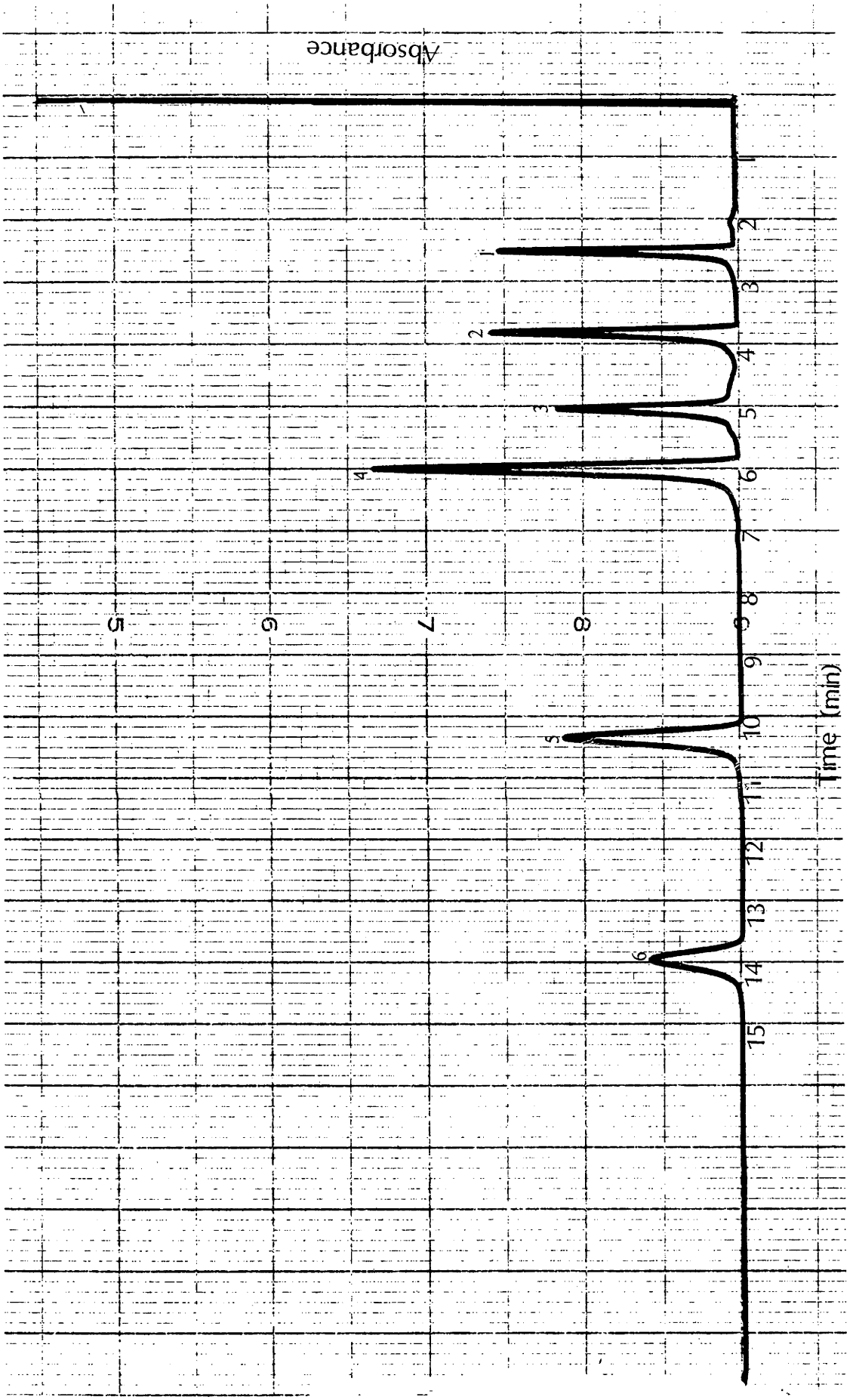

Absorbance
Time (min)
1
2
3
4
5
6

Figure 7-2: High pressure liquid chromatograph of synthetic β-carotene (2% 2-propanol in the solvent gradient described in the text, peak no.1 = β-carotene)

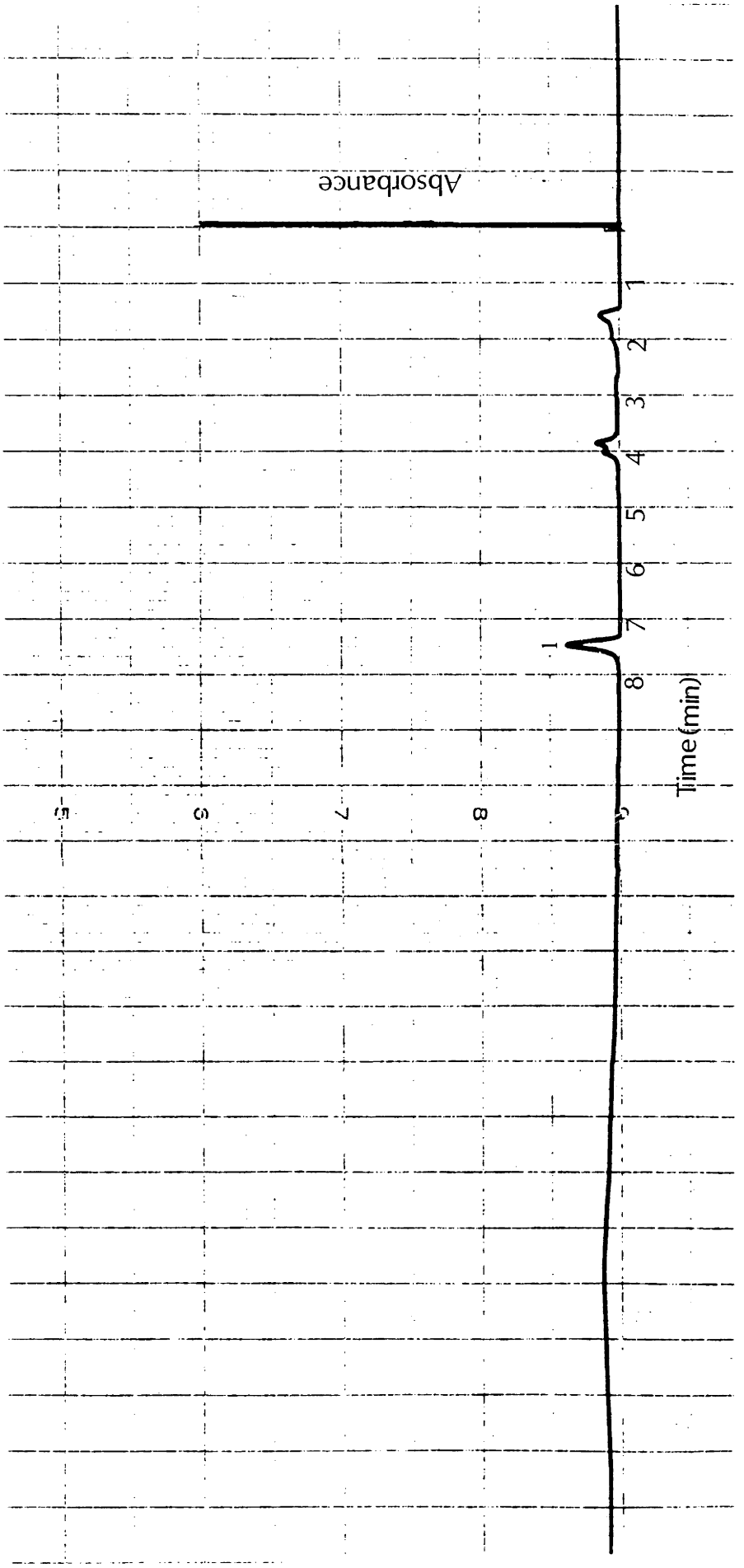

Absorbance
1
2
3
4
5
6
7
8
Time (min)

Figure 7-3: High pressure liquid chromatograph of synthetic β-carotene (4% 2-propanol in the solvent gradient described in the text, peak no.1 = β-carotene)

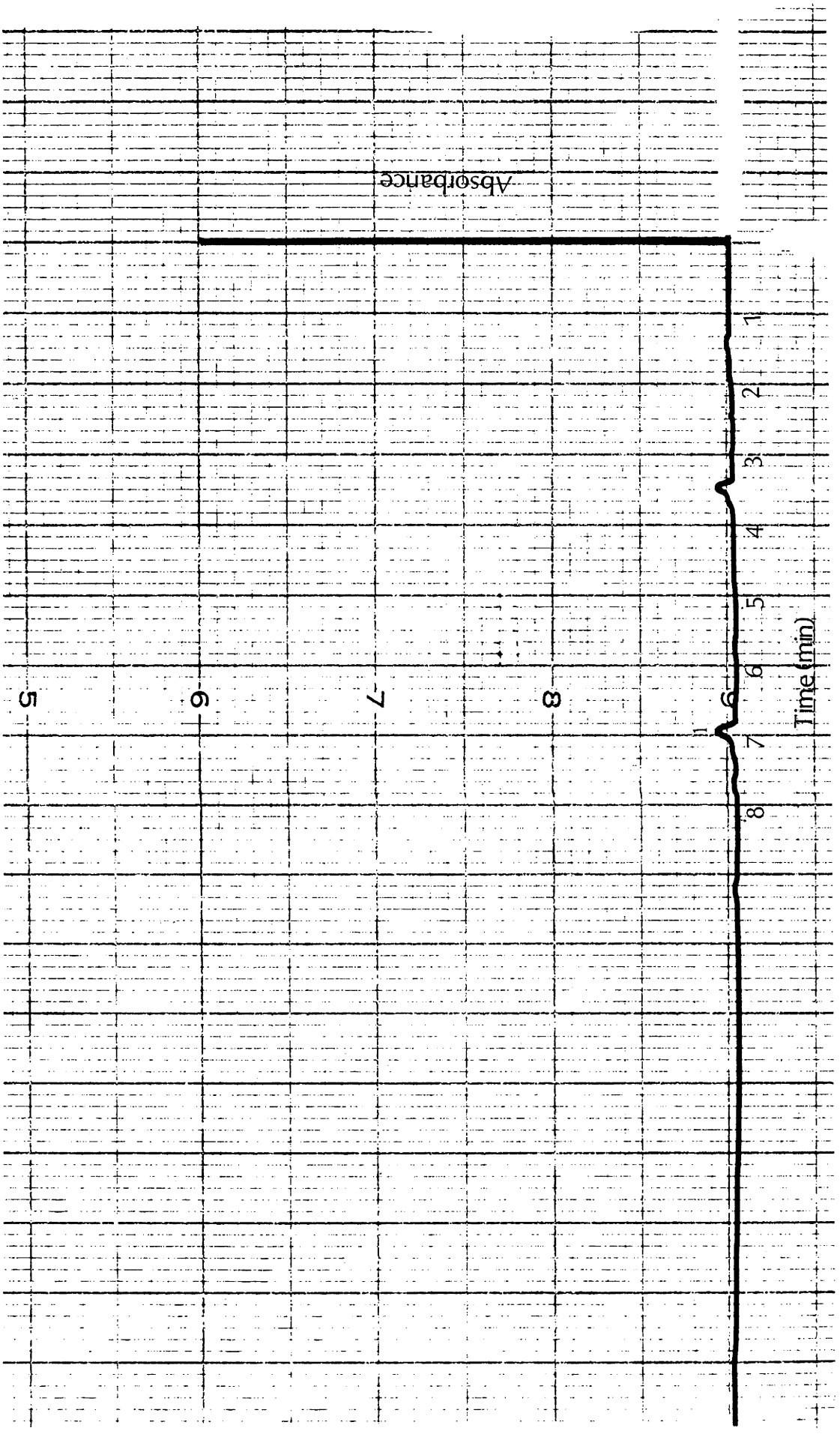

Absorbance
Time (min)

Figure 7-4: High pressure liquid chromatograph of synthetic β-carotene (7% 2-propanol in the solvent gradient described in the text, peak no.1 = β-carotene)

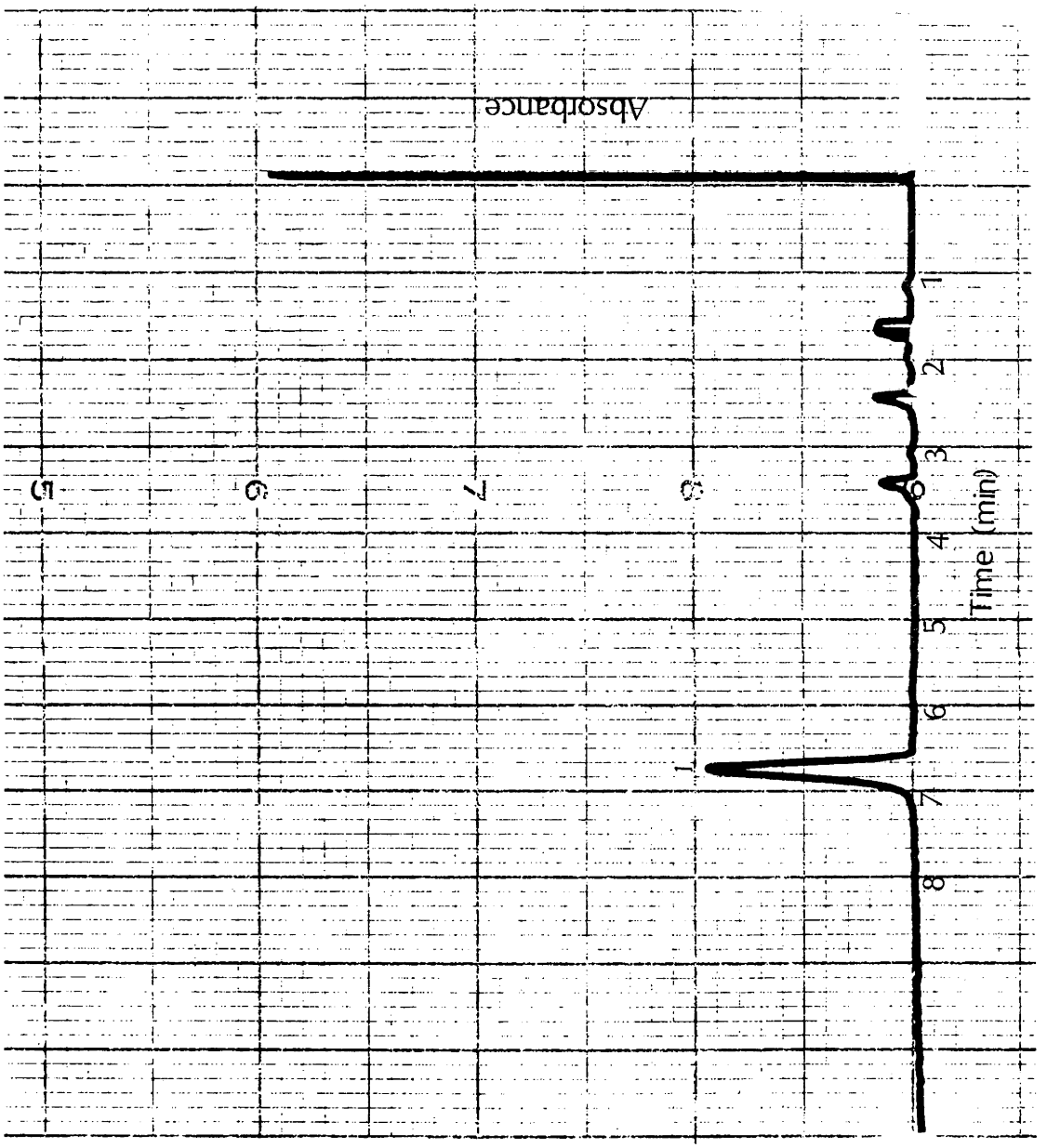
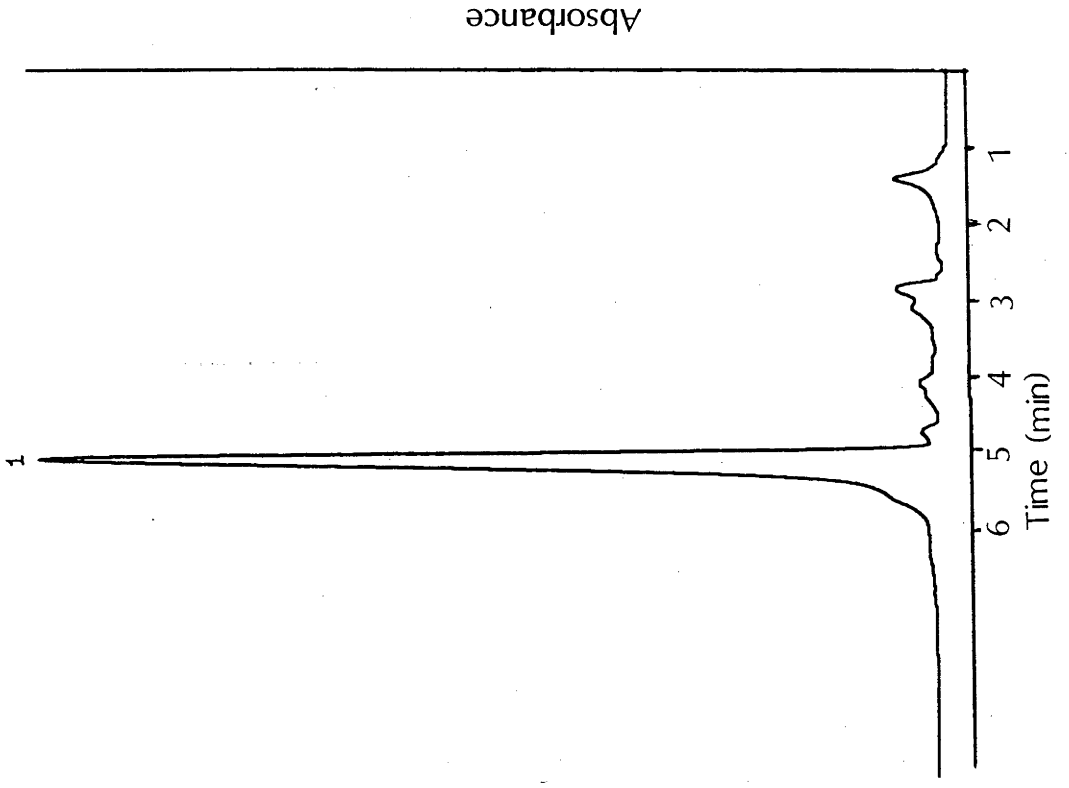

Absorbance
Time (min)
1
2
3
4
5
6
7
8
9
10

Figure 7-5: High pressure liquid chromatogram of synthetic β-carotene (9% 2-propanol in the solvent gradient described in the text, peak no.1 = β-carotene)

Absorbance
Time (min)
0
1
2
3
4
5
6
7
8
9
10
1
2
3
4
5
6
7
8

Figure 7-6: High pressure liquid chromatograph of synthetic β-carotene (10% 2-propanol in the solvent gradient described in the text, peak no.1 = β-carotene


Absorbance
1
1
2
3
4
5
6
Time (min)

7.3.3. Calibration of recorder

Figure 7-7 depicts the linear relationship between the concentration of β-carotene solution and peak area. It was found that the linear relationship of concentration and peak area was maintained in the entire working range (from 0 to 300 ppm).

7.3.4. Chromatography of yeast carotenoids

Figure 7-8 shows a typical chromatograph that results in the HPLC analysis of yeast carotenoids. Absorption spectra confirmed that the peaks number 1, 2, and 3 were torularhodin, torulene and β-carotene respectively. For the confirmation of the peak suspected as that of β-carotene, the absorption spectra of the third peak eluent and commercial β-carotene were compared (Figure 7-9). The major peak at 450 nm and the minor peak at around 460 nm confirmed that the third peak eluent was β-carotene. The retention time of β-carotene obtained from yeast extracts corresponded exactly to that of the standards analysed under same conditions. Minor variations in retention times among individual analyses were observed. Table 7-1 shows mean retention times and standard deviations in five consecutive separations and relatively minor error involved in the calculation of the capacity factor (k'). Table 7-2 depicts estimates of the components of the yeast carotenoids complex separated by HPLC and in absolute terms and as percentages of total. Cultures subjected to this analysis had been grown for 3 d at 25°C and hence the high β-carotene content. The amount of total carotenoids biosynthesized appear to be normal under these cultural conditions.

7.3.5. The effect of age of culture on carotenoids biosynthesis

Table 7-3 shows the effect of age of culture on the biosynthesis of the total carotenoids, β-carotene and torularhodin. The total carotenoids complex biosynthesized increased dramatically from the first to the third day of culture. This increase was also accompanied by the β-carotene content, but not by torularhodin. The increases in the total carotenoids from the third to the fifth and fifth to the seventh day were moderate. In the case of β-carotene nearly a 50% reduction from the third to the seventh day was observed. Torularhodin content underwent almost a tenfold increase from the third to the seventh day after culture. After 10 days, accumulation of torularhodin continued while the total carotenoids content and β-carotene content marginally declined. As a percentage of the total carotenoids, torularhodin content increased from 3.5% to 89.1% during the period under investigation. Correspondingly, β-carotene content underwent a reduction from 36.2% to 7.5% during the same period.

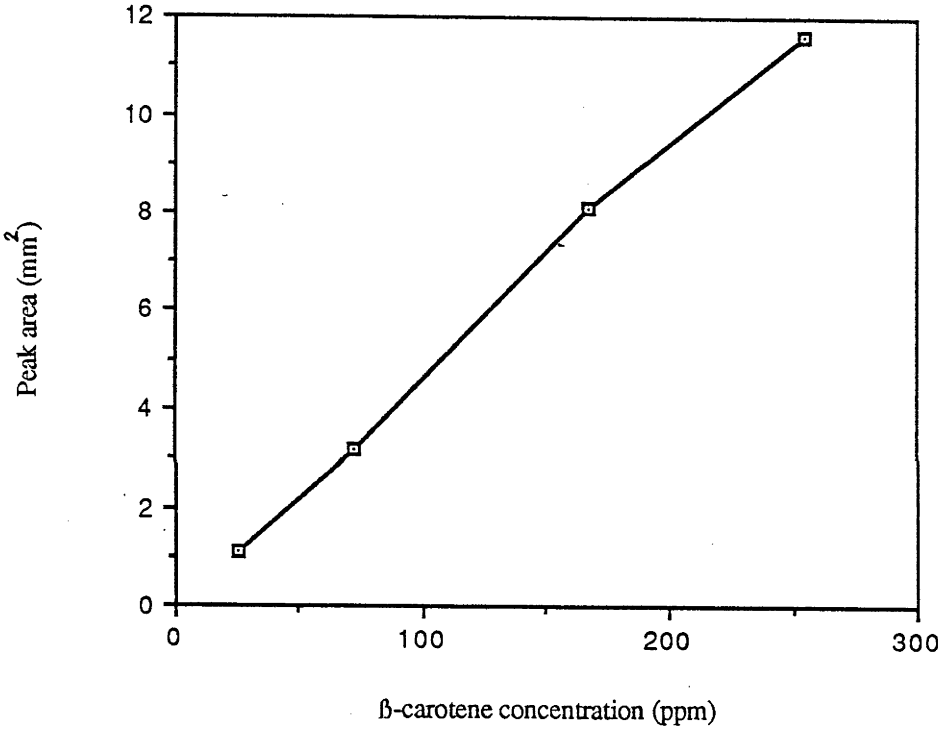
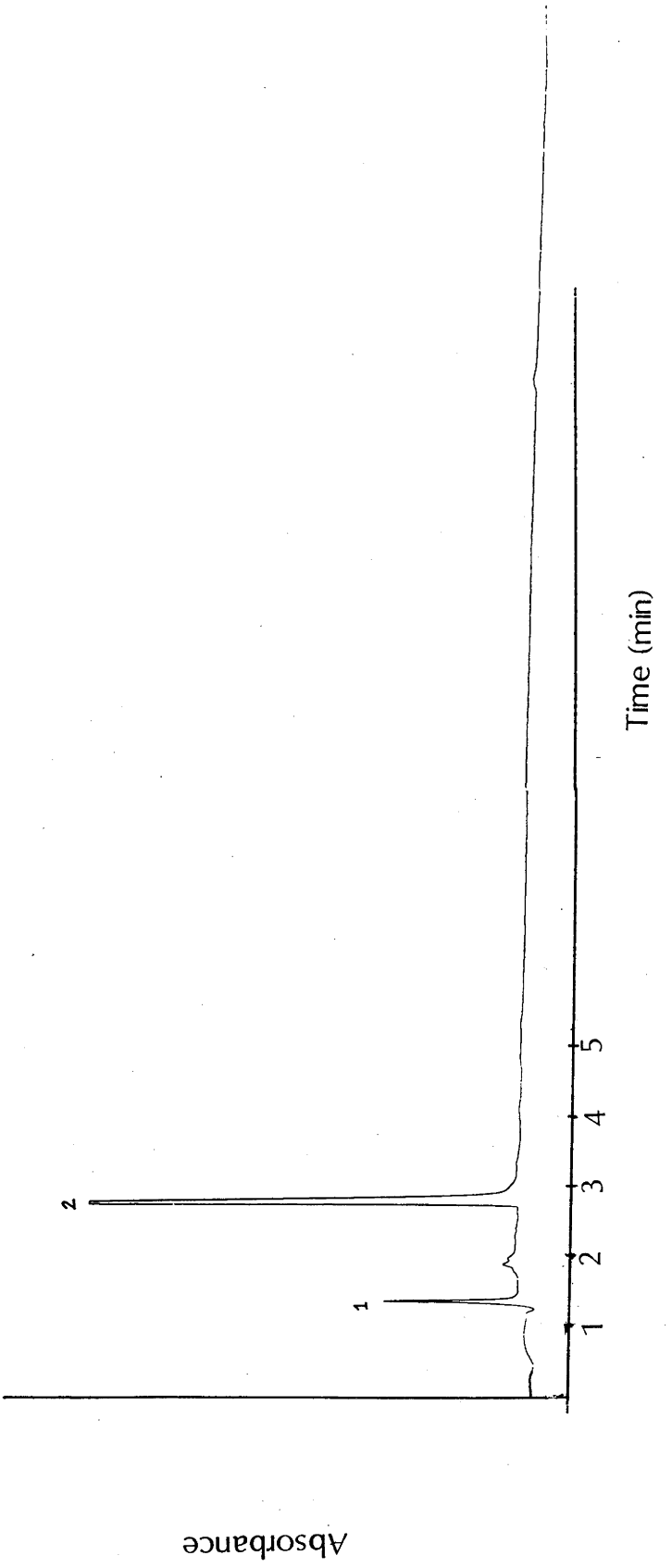


Figure 7-7: Relationship between β-carotene concentration and chromatograph peak area

Figure 7-8: High pressure liquid chromatograph of the yeast carotenoids (Recorder speed = 0.5 cm/min, peak no.s 1 and 2 = torularhodin and β-carotene respectively. Chromatographic conditions are described in the text)


Absorbance
Time (min)
1
2
1
2
3
4
5

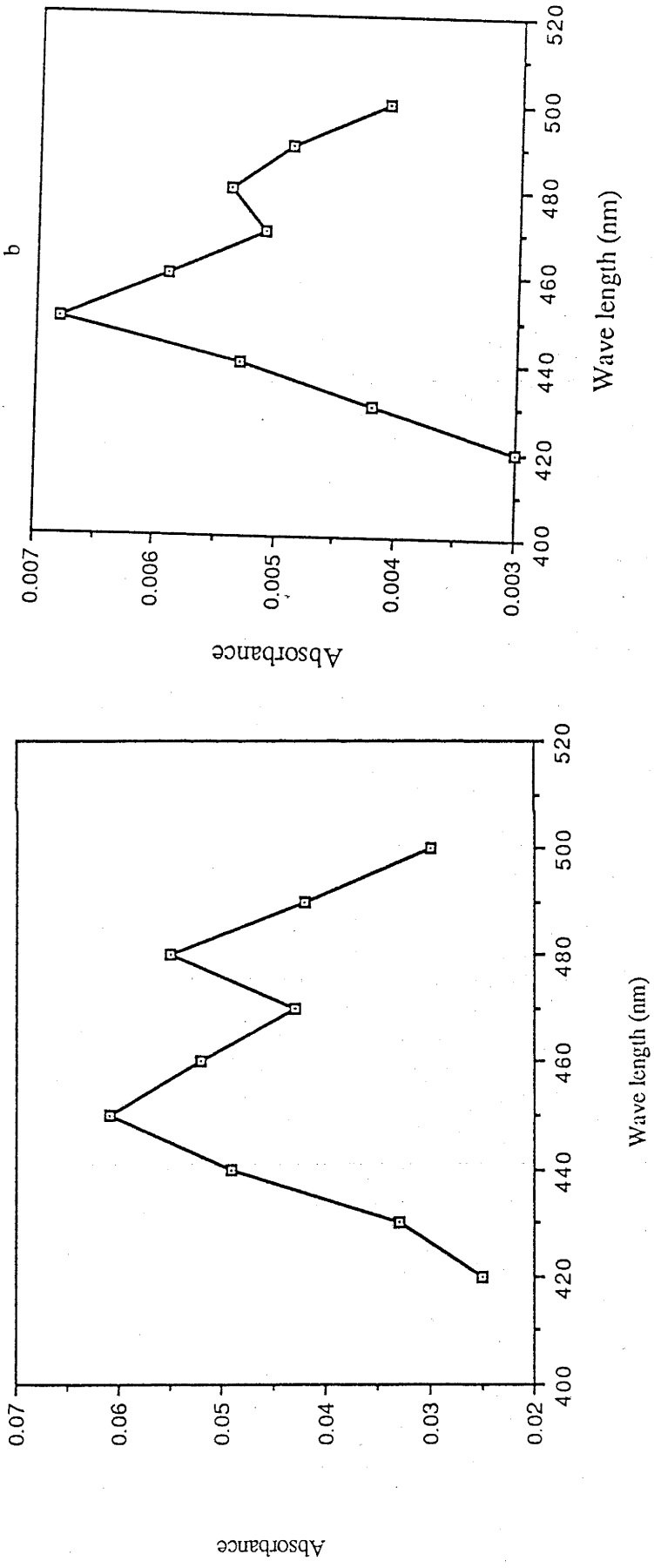


Figure 7-9: The absorption spectra of β-carotene and the peak eluent (3).
a = β-carotene, b = peak no.3

Pigment	Retention time (seconds)					Mean r.t.+S.D.	k'+S.D.
	1	2	3	4	5		
Torulene	304	308	299	310	306	305.4 ± 4.21	3.55 ± 0.06
Torularhodin	242	244	240	246	240	242.4 ± 2.6	1.50 ± 0.56
β-carotene	852	882	864	870	834	860.4 ± 36.5	11.78 ± 0.23

k' = Capacity factor, S. D. = Standard deviation

Table 7-1: Retention times (in s) of carotenoids during five successive separations under identical conditions

Pigment	µg/g Fresh weight of yeasts	% Total
β–carotene + γ–carotene	102.7 ± 3.4	45.0
Torulene	79.8 ± 2.6	35.0
Torularhodin	45.6 ± 1.5	20.0
Total	228.1 ± 4.7	

Table 7-2: Composition of the yeast carotenoids complex as determined by HPLC

Table 7-3: The effect of age of culture on carotenoid biosynthesis

Age of culture	Total carotenoids content (μg/g)	*b*-carotene content (μg/g)	Torularhodin content (μg/g)	*b*-carotene % total	Torularhodin % total
1 Day	162.4	58.7	5.6	36.2	3.5
3 Day	294.5	210.4	22.4	71.4	7.6
5 Day	311.8	223.7	96.3	71.7	30.8
7 Day	342.9	120.3	201.9	35.0	58.8
10 Day	348.7	26.1	310.6	7.5	89.1

7.3.6. The effect of temperature on carotenoids biosynthesis

Table 7-4 depicts the influence of temperature of incubation on carotenoids biosynthesis. The contents of total carotenoids, β-carotene, torulene and torularhodin biosynthesized increased with increasing temperature. The increase from 5°C to 15°C was marginal. At 25°C, a very significant increase in carotenoids biosynthesis over 15°C was observed. However, the β-carotene content as a percentage of the total pigments declined with increasing temperature, suggesting a tendency to biosynthesize more at lower temperatures. Contrastingly the torularhodin content increased at higher temperatures while torulene content remained constant.

7.3.7. The effect of C:N ratio on carotenoids biosynthesis

Results of the experiment on the effect of varying ratios of carbon and nitrogen on the biosynthesis of carotenoids are given in table 7-5. An increase of total carotenoids biosynthesis at all levels of carbon and nitrogen, than in the basal medium could be observed, though of varying proportions. The highest level of biosynthesis was observed when the basal medium was supplemented with both C (glucose) and N (yeast extract) at the highest level used (5%). However, when glucose and yeast extract were compared, glucose seemed to stimulate carotenoids biosynthesis more than yeast extract. The effect of 2% glucose was more beneficial than 5% yeast extract. The content of β-carotene followed the same trends.

7.3.8. The effect of illumination on carotenoids biosynthesis

Table 7-6 depicts the effect of illumination. Illumination during the first 6 h of culture appears to be critical for the biosynthetic process. While darkness dramatically reduced accumulation of the total carotenoids and all components tested, the difference between illumination during the first 6 h and during the total period of culture was not significant. This observation was common to β-carotene, torulene, torularhodin and the total pigments complex.

Carotenoid content (µg/g dry weight)				
	β-carotene	Torulene	Torularhodin	Total carotenoids
5°C	114.5	32.4	17.4	164.3
15°C	131.1	36.7	81.7	249.5
25°C	159.9	40.1	206.8	406.8

Table 7-4: The effect of temperature on carotenoids biosynthesis

Medium	Total carotenoids content (µg/g)	β–carotene content	
		µg/g	%
Control (Basal Medium)	147.4	34.7	23.6
BM* + 2% Glucose	177.6	46.1	26.0
BM + 2% YE#	151.6	28.6	18.9
BM + 5% YE	160.9	34.5	21.5
BM + 2% Glucose + 2% YE	193.0	67.1	34.8
BM + 5% Glucose + 5% YE	328.4	217.4	66.2

* = Basal medium, # = Yeast extract

Table 7-5: The effect of nutrition on carotenoids biosynthesis

	Carotenoid content (μg/g dry weight)			
	β-carotene	Torulene	Torularhodin	Total carotenoids
No illumination	147.8	40.1	89.8	277.7
Illumination	210.5	61.2	168.4	440.1
6h illumination	203.8	58.7	173.1	435.6

Table 7-6: The effect of illumination conditions on the biosynthesis of carotenoids in *Rhodotorula glutinis*

7.4. Discussion

The results suggest that reversed phase stationary phases are eminently suited for the quantitative determination of carotenoids. The rp-HPLC separation method described permits separation of the yeast carotenoids complex within the short analysis time of 15 min. The carotenoids mixture (carotenes and xanthophylls) separated differs from other reported mixtures that have consisted solely of either xanthophylls (Fiksdahl *et al.*, 1978; Braumann and Grimme, 1981) or carotenes (Will and Ruddat, 1985). Chromatographic conditions for the HPLC analysis of a mixture of polar and nonpolar carotenoids such as the yeast carotenoids have not been reported earlier. This study reports a HPLC separation of yeast carotenoids on a C_{18} reversed phase column with 2-propanol, acetonitrile-water gradient. Considering the limitations in sensitivity, length of analysis time and decomposition of carotenoids associated with conventional chromatographic methods, rp-HPLC proves to be a significant progression. Insufficient sensitivity of conventional chromatographic methods have been demonstrated by their inability to detect all components in mixtures, particularly those present in traces. Thin layer chromatography employed in the analysis of the pigment complex of an orange strain of the smut fungus *Ustilago violacea* recorded the presence of only γ-carotene (Garber *et al.*, 1975). The same extract when subjected to open column chromatography on alumina was found to contain lycopene, phytoene and neurosporene. However, HPLC detected all the carotenoids contained in this extract, consisting of lycopene, phytoene, neurosporene, γ- carotene and β-carotene. Will *et al.*, (1984) using HPLC found that a yellow strain of *Ustilago violacea* synthesized phytoene, ζ-carotene, neurosporene, β-zeacarotene and β-carotene. Garber *et al* (1975) had earlier reported after a TLC analysis, that this strain of the fungus synthesized only β-carotene. Dangers of such exclusions of important components of a mixture can be eliminated by resorting to HPLC. The registering of new compounds is made possible by the extreme sensitivity of HPLC to compounds present in minute quantities. This extreme sensitivity also enables detection of small variations of the physico-chemical properties of the structures of the compounds concerned. Braumann and Grimme (1981) demonstrated this capability by separating structurally related herbicides using reversed phase HPLC. This is probably due to the characteristics of the bonded phase silica which shows quasi adsorptive properties (Braumann and Grimme, 1981). Retention in rp-HPLC therefore does not seem to be due to the interactions of the solutes with the adsorbent surface, but the effect of the solvent which directs solute molecules to the C_{18} ligands. Consequently, the composition of the mobile phase plays an important role in

selectivity. Apart from sensitivity, the rapidity of analysis makes HPLC superior to the conventional chromatographic techniques. The fastest of the conventional techniques, TLC, is rarely quantitative while the more quantitative open column chromatography is slow, with an average analysis time of 2-3 h. Exposure to environment in systems such as open columns for long durations allow photoisomerization, oxidation and breakdown of carotenoid molecules. Reduced analysis time and closed experimental system (column) in HPLC drastically reduce carotene degradation and the appearance of breakdown products. The weak hydrophobic interactions that execute separations in reversed phase systems also ensure that labile structures of carotenoids are not transformed.

Comparison of the present results and the analyses of microbial carotenoids reported by Will *et al.* (1984) and Will and Ruddat (1985) illustrate that the conditions of analysis cause substantial differences in retention times for a given compound. Even though the carotenoids separated in those studies originated from *Ustilago violacea*, and therefore lacked the diversity of the yeast carotenoids complex, these separations are comparable in having separated β-carotene from a mixture of carotenoids. Will *et al.* (1984), using a C_{18} column and a 2-propanol, acetonitrile-water gradient separated six carotenes including β-carotene with a comparatively long retention time of 37 min for β-carotene. Identical retention times have been obtained by Will and Ruddat (1985) who used an octadecylsilane (ODS) column with the same solvent gradient. Inexplicably, the use of the C_{18} column (of lower hydrophobicity than the ODS column) has not recorded a decrease in the retention time. The C_{18} column and the chromatographic conditions employed in the present analysis have reduced retention times by more than 50%. However, the column characteristics may be fundamentally important in determining retention times. The number of theoretical plates of the C_{18} column employed by Will *et al.* (1984) has been only 10 000. The Alltech C_{18} column used in the present experiment with a theoretical plate equivalent of 26 000, denotes a more than 100% increase in efficiency and resolution. The number of theoretical plates therefore is a reliable indicator of the efficiency of a column. Further, the initial operational pressure of the present analysis (11 MPa) is nearly twice that used by Will *et al.* (1984). The potential for shortening retention times further by increasing the amount of 2-propanol in the initial gradient could not be exploited in this experiment. Limits to the amount of 2-propanol in the solvent mixture were imposed by torularhodin which, due to its high polarity, is not retained adequately at high levels of 2-propanol. Separation of torularhodin prior to HPLC by phase separation affords an opportunity of shortening retention times. However, separation of the total pigment

complex by HPLC yielded more accurate results. The shortening of column life, caused by pressure increases accompanying increases in the proportion of 2-propanol also has to be taken into consideration.

Analysis of the yeast pigments complex, biosynthesized after 3 d culture at 25°C show that these conditions are ideal for the biosynthesis of β-carotene which constituted approximately 45% of the total pigment complex. Similar observations were earlier made with regard to the total carotenoids complex. This could not be confirmed in relation to the other components due to detection of eluents at 450 nm, excluding them from the analysis. The chromatograms yielded in this series of experiments demonstrated a high degree of reproducibility. High reproducibility of chromatographic separations (characterised by low standard deviations of retention times and capacity factors) is a feature of HPLC that is not unique to this analysis. In this respect HPLC is vastly superior to the conventional methods of chromatography. Absorption spectroscopy as used here is the most widely used method of identifying carotenoids. The specific, solvent dependant absorption spectrum that consist of three sharp peaks at characteristic wavelengths make carotenoids ideal compounds for the application of this method. However, modern methods of detection and quantification of separated compounds have been developed recently. The detection methods include the use of changes in bulk solute properties such as refractive index whereas quantification is achieved by internal integration.

The adoption of rp-HPLC has enabled rapid examination of the effects of cultural age, temperature, nutrition and illumination on the biosynthesis of β-carotene in *R. glutinis*. These results suggest that the effect of cultural factors on the biosynthesis of β-carotene is different to the effects on the biosynthesis of the total carotenoids complex and torularhodin. The effects of cultural conditions on total carotenoids biosynthesis presented in chapter 4 are reasserted here. The trends in biosynthesis of β-carotene suggest that this compound is an intermediate compound in the pathway of carotenoids biosynthesis, of which torularhodin is the ultimate product. Such a conversion is compatible with the pathway proposed by Simpson (1972). A temperature sensitive system seems to be channelling intermediates either to β-carotene or torularhodin. This process also seems to be species dependant. Nakayama *et al.* (1954) reported that the β-carotene content of *R. peneaus* was greatly reduced when grown at low temperatures, whereas β-carotene accumulation in *R. rubra* was unaffected by temperature.

Increased age of culture and high temperature seem to accelerate the conversion of

β-carotene to torularhodin. A decrease in the β-carotene content (both in absolute and percentage terms), accompanied by an increase in torularhodin content, is evident of such a trend. Further in old cultures the total carotenoids complex appears to consist almost entirely of torularhodin. In up to 5 d old cultures, these two pigments constituted only around 60% of the total complex. This suggests that the process of pigment biosynthesis occurs in two steps: i) biosynthesis of β-carotene and its precursors, ii) conversion of these compounds to torularhodin. High levels of nutrition appear to be generally conducive to pigment biosynthesis. High quantities of carbon is a prerequisite for increased biosynthesis of β-carotene. This is probably due to the need for growth prior to the initiation of the biosynthesis of pigments. However, excess nitrogen undoubtedly increases biosynthesis when coupled with high levels of carbon. The effect of illumination on carotenoids biosynthesis seems to be one of initiating it rather than a qualitative or a quantitative effect on the process when its underway. The results indicate that total illumination does not increase the synthesis of either the total pigments complex or any of its components over the initial 6 h period of illumination. This suggests that once the process is triggered off by exposure to light, it continues irrespective of the subsequent conditions of illumination.

HPLC is particularly useful in investigations of the effect of cultural conditions on the biosynthesis of a single component of a pigment complex. In such studies, the large number of analyses involved makes reliance on slow analytical techniques (e. g. open column chromatography) impractical. Also the reproducibility of an analysis is of extreme importance. HPLC provides a rapid, sensitive, and more accurate method for such analyses. Based on HPLC analysis, a genetic model for carotenogenesis in the smut fungus *Ustilago violacea* has been proposed (Will and Ruddat, 1985).

Chapter 8

Environmental analysis of the biosynthesis of carotenoids in the tea leaf

8.1. Introduction

The carotenoids are the most widely distributed natural pigments found in higher plants. All plant tissues which contain chlorophylls have been found to contain carotenoids (Goodwin, 1965). The carotenoids complex occurring in green plant tissues consists of β-carotene, lutein, violaxanthin and neoxanthin as its major components. Antheraxanthin and zeaxanthin occur as minor components. In spite of its ubiquity the carotenoids have not been assigned a definite role in plant metabolism. The efficient transferrence of light energy to chlorophyll for utilization in photosynthesis has earned it the role of a secondary photoreceptor (Haxo, 1960). This role was refuted by Yamamoto and Chichester (1965) who concluded that the carotenoid pigments do not play a direct role in oxygen transport in photosynthesis. It has been suggested that the carotenoids protect chlorophylls in photosynthesizing tissues against photosensitized oxidation (Wallace and Schwarting, 1954; Krinsky, 1966). The colour imparted by the carotenoids to flower petals, reproductive structures and fruits is thought to possess an insect attractant or repellant effect. In tea, leaf carotenoids have assumed a special role by being associated with the quality of black tea. An association between the tea leaf carotenoids content and the seasonal incidence of flavour in tea has been long speculated (Tirimanne and Wickremasinghe, 1965). Proof of such an association was provided by Sanderson *et al.* (1971) and Sanderson and Graham (1973). These workers reported that β-carotene is oxidatively degraded to a number of volatile and nonvolatile aroma compounds during black tea manufacture. Wickremasinghe (1974) explained the seasonal nature of flavour on the basis of increased biosynthesis of carotenoids in the tea leaf via an extra-chloroplastidic pathway. This suggests an environmentally controlled pathway of carotenoids biogenesis.

Bright, dry weather and low-moisture soil conditions are believed to stimulate the biosynthesis of leaf carotenoids (Bickford and Dunn, 1972; Wickremasinghe, 1974). The investigations on the effect of light on the process of carotenoids biosynthesis have

revealed qualitative and quantitative effects. The carotenoids of dark-grown, etiolated seedling leaves mainly consist of the xanthophylls lutein and violaxanthin with β-carotene constituting only 3-9% of the total carotenoids content (Lichtenthaler, 1967; 1969). Upon exposure of etiolated seedlings to light, Lichtenthaler (1969) observed a rapid accumulation of β-carotene during the first 24 h period of chlorophyll formation. The final proportion of β-carotene in the fully developed leaf appears to depend on light intensity and is generally higher under high light intensities (Lichtenthaler, 1979). Wheat seedlings grown under light contained approximately twice as much carotenoids as dark grown plants of equal age. The light grown plants produced a nine-fold increase in carotenes (Wolf, 1963). A comparative analysis of the carotenoids of 14 crop species during summer and winter by Takagi (1985) showed that the carotenes constituted 30-50% of the carotenoids complex biosynthesized during summer, but declined to 17-34% in winter. The quantity of total carotenoids biosynthesized did not exhibit significant differences between the two seasons. In an analysis of leaf carotenoids of a number of tea clones grown in Assam, India, the minimum and maximum carotenoids concentrations were recorded during the rainy month of July and dry April respectively (Hazarika and Mahanta, 1984). Ullah (1979) observed that carotenoids vary quantitatively in different parts of the tea shoot. This study reported that the bud contained the minimum quantity of β-carotene while a sequential increase was observed from the first to the second leaf.

The intimate association between chlorophylls and carotenoids in plant tissues often necessitates simultaneous analysis of plant extracts (Braumann and Grimme, 1979). However, separate analysis of leaf carotenoids involves basic hydrolysis of lipids or esters of xanthophylls (saponification) followed by chromatography. The majority of the reported analyses of tea leaf carotenoids have employed thin layer chromatography (TLC) as the method of analysis (Tirimanne and Wickremasinghe, 1965; Ullah, 1979; Hazarika and Mahanta, 1983; Hazarika and Mahanta, 1984). This technique is often criticized for exposing samples to the atmosphere leading to oxidation and photoisomerisation. In all reported separations of carotenoids with TLC, silica gel has been used as the stationary phase either alone or in combination with another compound. Tirimanne and Wickremasinghe used silica gel for the separation of carotenes, and kieselguhr and oil for the separation of xanthophylls. Ullah (1979) used silicagel as the stationary phase with different solvent systems for the separations of carotenes and xanthophylls. Ether or acetone, and 25% acetone in light petrolium were used in the separation of carotenes and xanthophylls respectively, after an initial separation of carotenes from xanthophylls with

1% ether in light petroleum. Hazarika and Mahanta (1983, 1984) used a 2:1 mixture of cellulose and silica gel as stationary medium and benzene, petroleum ether, ethanol and water (10:10:2:1) as solvents.

Recent reports of applications of high pressure liquid chromatography (HPLC) in the analysis of green leaf carotenoids (Braumann and Grimme, 1981; Takagi, 1985) suggest that this technique could facilitate the faster and more sensitive separations of tea leaf carotenoids. In these analyses 10 μm reversed phase C_8 column with a 25:75 linear gradient of methanol and acetonitrile (Braumann and Grimme, 1981), and an unspecified stationary medium with hexane-benzene and acetone as solvents (Takagi, 1985) have been used. These analyses are considerably faster than those employing column and thin layer chromatography and involve less handling.

8.2. Materials and Methods

8.2.1. Carotenoids sample preparation for the HPLC analysis

Extraction of tea leaf carotenoids: Tea leaf sample was cut into pieces and ground vigorously in a mortar after adding 25 ml portions of acetone. The extraction was continued until the remaining leaf mass was devoid of any pigments. The acetone extract was made to volume in a 250 ml volumetric flask and transferred to a separating funnel. The lipid soluble fraction was extracted in diethyl ether by adding an equal volume of diethyl ether and water until the two layers separated. The extract in diethyl ether was washed three times with water and filtered through Na_2SO_4.

Saponification: The extract in diethyl ether was later dried in a slow stream of nitrogen and dissolved in 25 ml of ethanol. The carotenoids solution in ethanol was saponified by adding 10 ml of KOH (60% w/v) and allowing to stand under nitrogen at 5°C overnight. Carotenoids were taken up in diethyl ether by adding an equal volume of the same and a dilute NaCl solution until two layers were formed. Etheral extracts were washed with water until free from alkali.

Removal of sterols: Sterols were removed by refrigerating overnight at 0°C.

Aliquots for HPLC: Carotenoids were dissolved in the initial phase of solvents (10:90 of 2-propanol and acetonitrile-water(90:10)) and filtered through an 0.45 μm pore size Acrodisc filter (Gelman Sciences, Ann Arbor, Mi, USA) mounted on a syringe. Samples of

25 µl of the filtered solution was injected into the HPLC column with a micro-syringe. Procedures involved in the preparation of samples of tea leaf carotenoids are schematically presented in figure 8-1.

8.2.2. HPLC of carotenoids

The HPLC system used consisted of a Spectra-Physics SP8700 solvent delivery system (Spectra-Physics corporation, Santa Clara, CA, USA), an Alltech 605RPC C_{18} column (Alltech Associates Inc., Deerfield, Illinois, USA), a Micromeritics 788 variable wavelength detector (Micromeritics, Norcross, Georgia, U.S.A) and a Waters model 730 data module recorder (Waters Associates, Milford, Massachusets, USA).

Solvents: The solvents used in the analysis, 2-propanol and acetonitrile were of the HPLC grade purchased from Waters Associate, Sydney. Distilled water was used as a solvent. All the solvents were filtered through a 0.45 µm filter connected to the end of the delivery lines and immersed in the solvent reservoires. All solvents were degassed with helium using the facility incorporated into the solvent delivery system.

Positional analysis of tea leaf carotenoids: The carotenoids content in the bud, and the first and second leaves from the shoot apex were analysed to examine qualitative and quantitative effects of the age of the leaf on the carotenoids content. Mature, dark green leaves from the main stem of the plant was also subjected to carotenoid analysis for the purposes of comparison. A sample (10 g) of leaves were taken in each case and pigments extracted and anlysed as described above.

The effect of illumination on β-carotene biosynthesis: Tea plants (grown in pots) were exposed to light of different intensities in growth cabinets maintained at 25°C and 50% relative humidity. Light intensities of 7, 15, 30 and 40 Wm^{-2} were obtained by using appropriate 'day light' fluorescent lights. After exposure to these light intensities for 16 h the shoots were collected and analysed. In each case 10 g leaf samples were analysed. The experiment was repeated three times.

The effect of the difference between the maximum and minimum temperatures: Plants similar to the above were placed in growth cabinets maintained at 50% relative humidity and a 12 h light cycle of 30 Wm^{-2}. The plants were exposed to four levels of temperature differences (25-23°C, 25-20°C, 25-17°C, 25-14°C) and the new shoots plucked after 7 d. Analysis was carried out according to the methods described above.

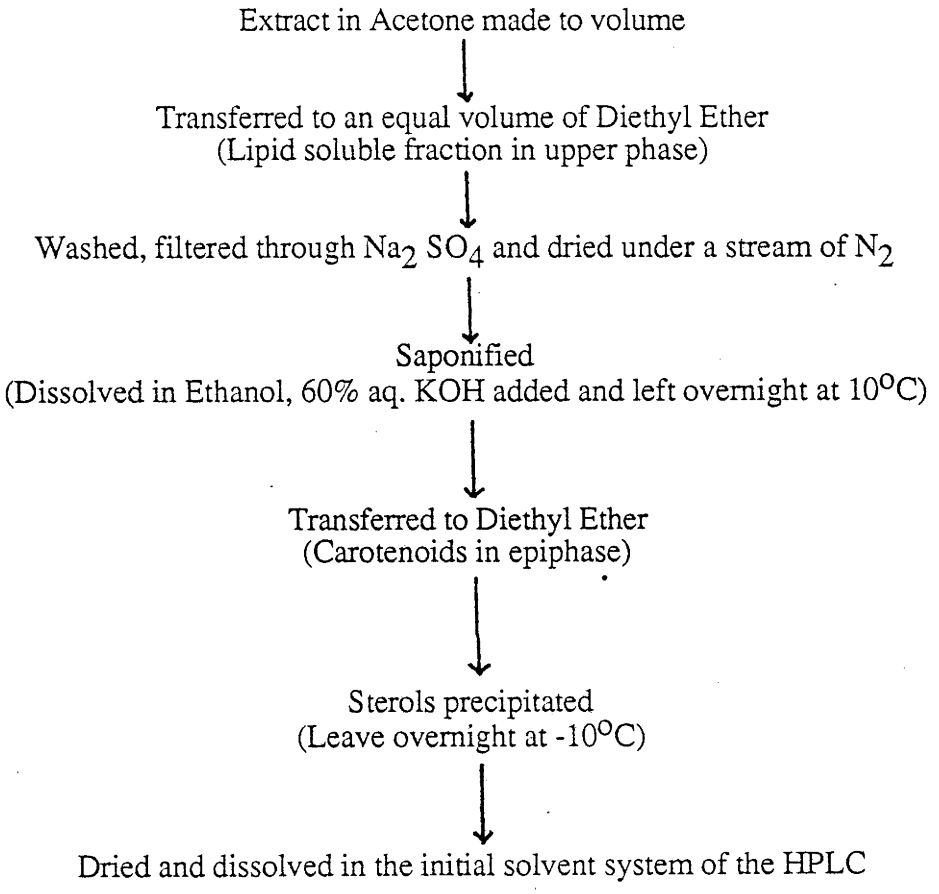


Figure 8-1: Major steps involved in sample preparation of tea leaf carotenoids for HPLC

8.3. Results

8.3.1. HPLC analysis of carotenoids

The chromatographic conditions developed for the separation of yeast carotenoids enabled a rapid and satisfactory analysis of the tea leaf carotenoids. Figure 8-2 shows the separation of carotenoids extract from the leaves (two leaves and the bud) of *Camellia sinensis* on an Alltech RP18 column routinely used for the analysis. Usually four peaks of major components of the carotenoids complex were observed. These peaks corresponding to peak numbers 1, 2, 5 and 6 were identified as neoxanthin, violaxanthin, lutein and β-carotene respectively by their absorption spectra. The two minor peaks (numbers 3 and 4) were suspected to be the minor components antheraxanthin and zeaxanthin. Though the peak eluents were inadequate for collection and examination of the absorption spectra for positive identification, their relative concentrations and the positions in relation to identified components suggest that these two carotenoids are antheraxanthin and zeaxanthin. As expected, the xanthophylls eluted before the carotenes in this reversed phase column. The retention time for β-carotene from the tea leaf extracts was identical to that from the yeast pigments. The retention time for neoxanthin, the first xanthophyll to elute, was 2 min longer than that for torularhodin. This is probably caused by the lower polarity of neoxanthin in comparison with torularhodin. The first carotene to elute was the peak number 6, β-carotene. Several minor peaks that appeared after this compound may be α-carotene and cryptoxanthin. The peak heights in this chromatogram could be regarded as true indicators of relative quantities of the different carotenoids contained in the tea leaf. Table 8-1 shows the identification data for the major peak eluents.

8.3.2. Positional analysis of tea leaf carotenoids

The chromatographs resulted from the rp-HPLC analyses of the carotenoids extracts from the bud, the first and second leaves from the shoot apex and mature leaf are given in figures 8-3 to 8-6. Comparison of the four chromatographs reveal the remarkable reproducibility of the retention times for each compound afforded by the HPLC. The peak numbers 1, 2, 3, 4, 5, and 6 refer to neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein and β-carotene respectively. The relative concentrations of all components of the carotenoids complex were highest in the bud. A dramatic reduction in the concentrations of all carotenoids in the first leaf (in comparison with the bud) was observed. The difference between the first and second leaves from the shoot apex was not significantly different

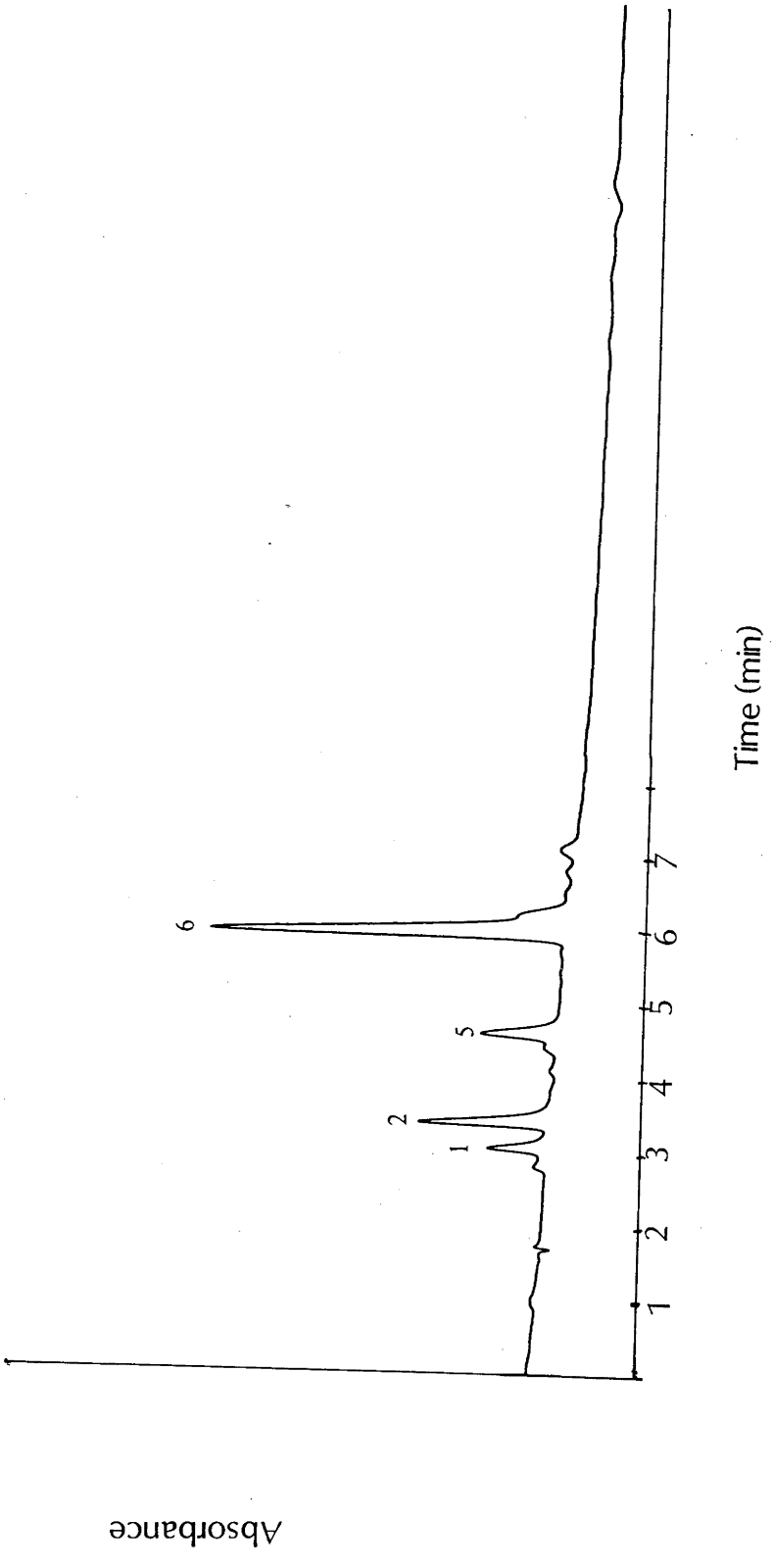

Absorbance
Time (min)
1
2
3
4
5
6
7
1
2
5
6

Figure 8-2: High pressure liquid chromatograph of tea leaf (two and the bud) carotenoids. Peak no.s 1, 2, 5 and 6 are neoxanthin, violaxanthin, lutein and β-carotene respectively

	Absorption maxima*					
	Observed values (nm)			Reported values# (nm)		
Neoxanthin	426	439	464	415	438	467
Violaxanthin	419	440	469	417	440	469
Lutein	420	444	472	422	445	474
β-Carotene	424	450	475	427	449	475

* = Carotenoids dissolved in ethanol, # = Davis, (1976)

Table 8-1: Identification data for the tea leaf carotenoids

Figure 8-3: High pressure liquid chromatograph of carotenoids of the tea shoot apex. (For peak identification refer Figure 8-2)

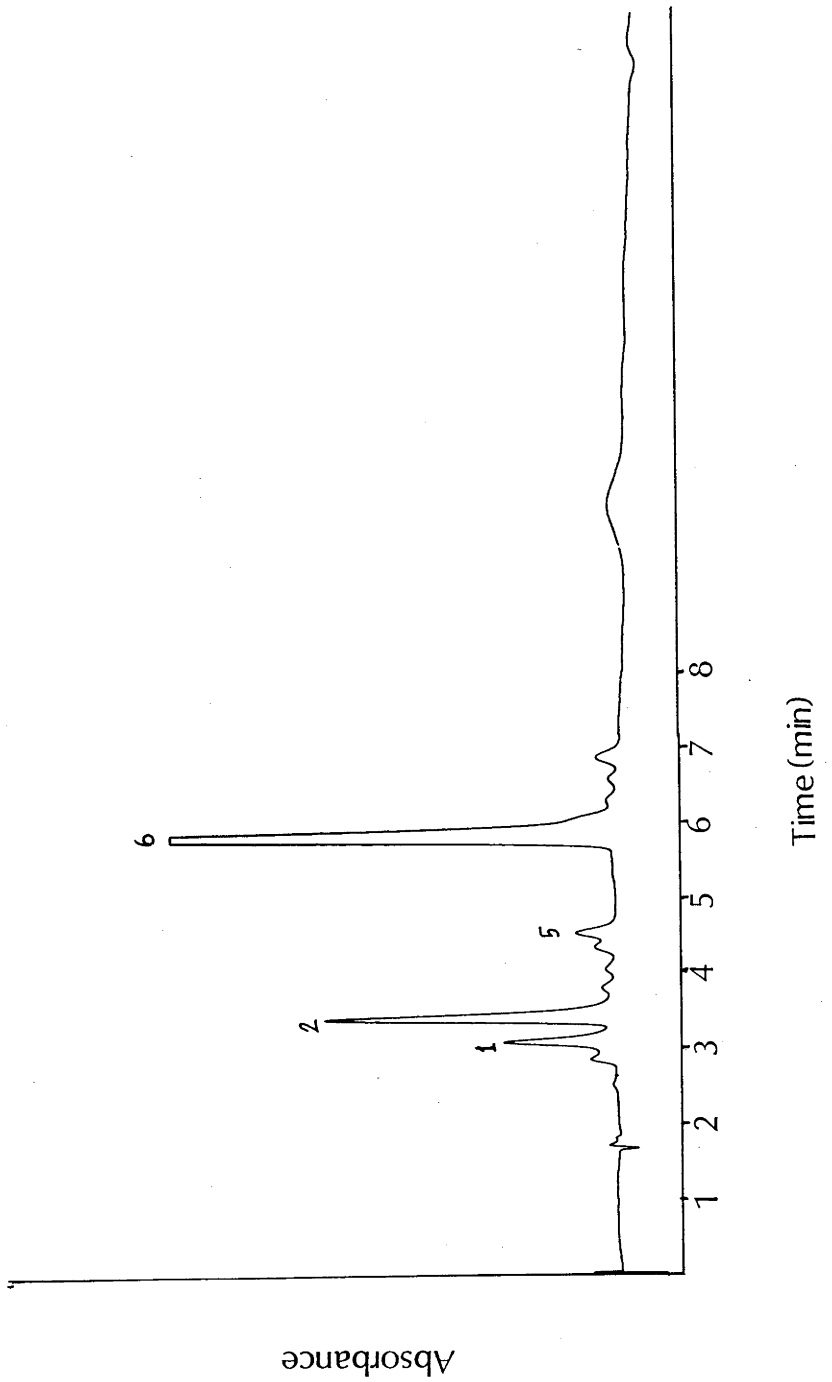

Absorbance
Time (min)
1
2
3
4
5
6
7
8
1
2
5
6

Figure 8-4: High pressure liquid chromatograph of carotenoids of the first tea leaf from shoot apex. (For peak identification refer Figure 8-2)

Figure 8-5: High pressure liquid chromatograph of carotenoids of the second tea leaf from shoot apex. (For peak identification refer Figure 8-2)

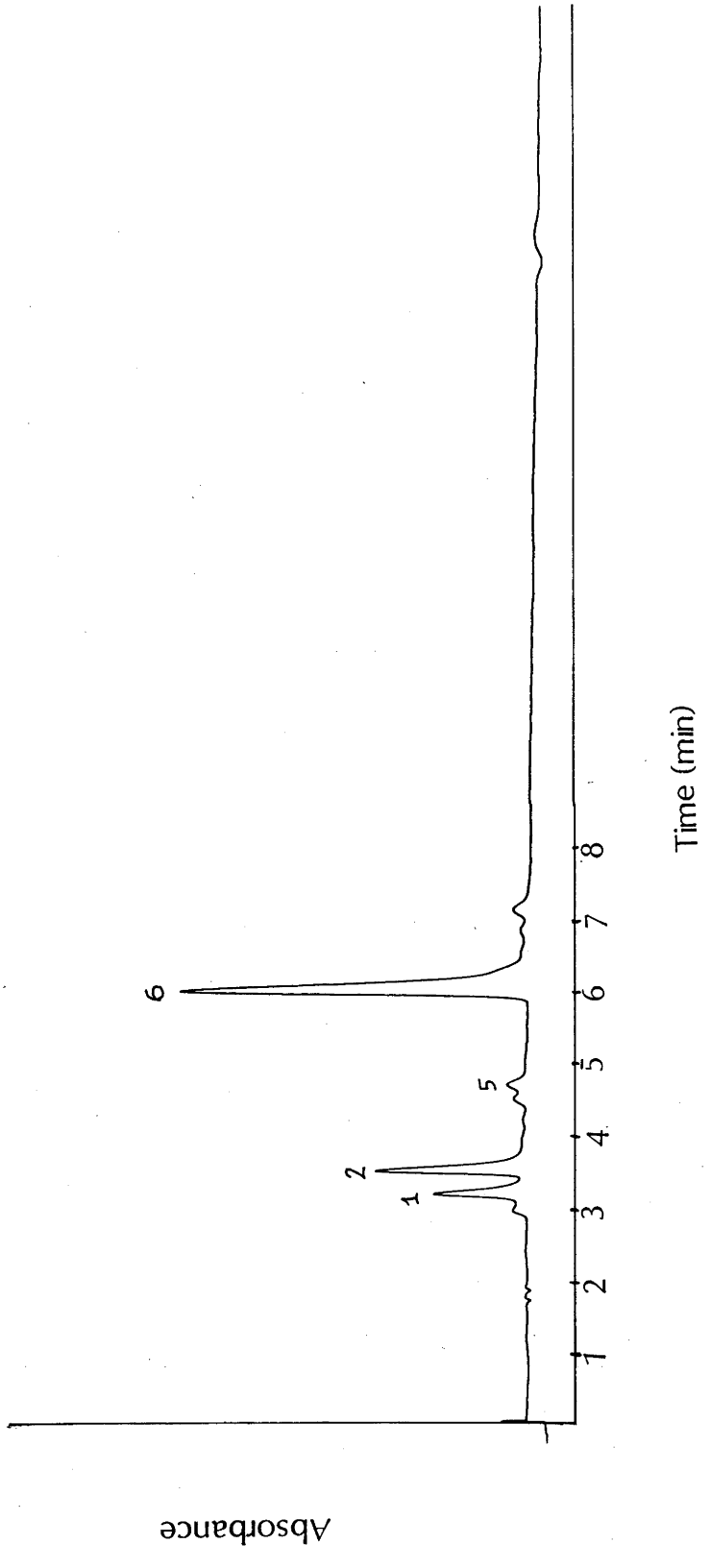
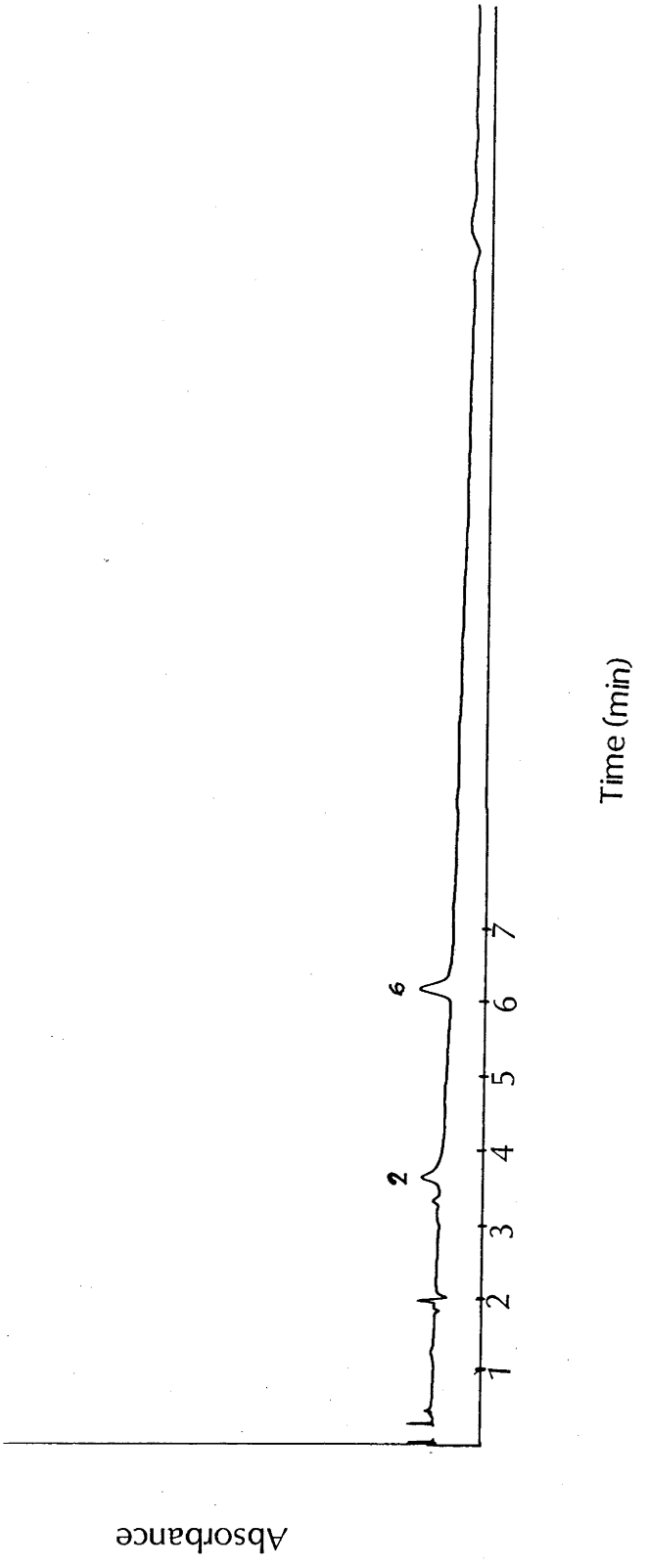

Absorbance
Time (min)
1
2
3
4
5
6
7
8
1
2
5
6

Figure 8-6: High pressure liquid chromatograph of carotenoids of the mature tea leaf. (For peak identification refer Figure 8-2)


Absorbance
Time (min)
1
2
3
4
5
6
7
2
6

	Carotenoid content (μg/g dry wt.)			
	Bud	First leaf	Second leaf	Mature leaf
Neoxanthin	34.6	23.2	36.9	-
Violaxanthin	79.3	52.8	40.2	6.9
Lutein	22.8	10.0	8.3	-
β-Carotene	114.9	83.1	71.1	15.6
Total carotenoids	251.6	169.1	156.5	22.5

Table 8-2: Relative quantities of major carotenoids contained in the bud, the first leaf and the second leaf of the tea shoot

($P<0.05$). However, the mature leaf contained only immeasurable quantities of carotenoids. The relative changes in individual carotenoids that accompanied leaf maturity were different with each component. A uniform reduction in the concentrations of all carotenoids was observed in the first leaf than in the bud. The relative decreases were highest in the major pigments generally with violaxanthin recording the highest reduction among these, followed by β-carotene and neoxanthin. Relative reduction in the minor components of the carotenoids were small compared to the decreases in the major pigments. The contents of violaxanthin and β-carotene in the first and second leaves from the shoot apex appear to be almost equal. However, the first xanthophyll to elute, neoxanthin, recorded a significant increase in the second leaf. This appeared to be the only difference between the carotenoids of the two leaves. The most dramatic reduction in the carotenoids content was found in the mature, dark green tea leaf sample. A total disappearance of the minor components antheraxanthin and zeaxanthin and a drastic reduction in the contents of neoxanthin, violaxanthin and β-carotene was observed. The concentrations of these carotenoids in the mature tea leaf were such that a less sensitive method of analysis would not have detected their presence. The quantities of carotenoids contained in the bud, the first and second leaves are presented in Table 8-2.

8.3.3. Effect of light intensity on carotenoids biosynthesis in the tea leaf

Figures 8-7 to 8-10 show the chromatographs of the carotenoids extracts of the tea leaves exposed to different intensities of light. Once again, the analyses proved the reproducibility of the separation method developed. Quantitative differences were observed in the carotenoids of the leaves exposed to different light intensities. A disappearance of the xanthophylls which occurred as trace amounts in the normal leaf samples, was observed. The carotene component was limited to β-carotene. The carotenoid content of the leaves maintained under complete darkness consisted predominantly of the xanthophylls neoxanthin, violaxanthin,and β-carotene. The minor components of the xanthophyll fraction, antheraxanthin and zeaxanthin did not occur in these leaves. The synthesis of only traces of lutein in the absence of light appears to be a significant effect of this factor, considering the relatively large quantities of lutein formed under normal light conditions. A general reduction in the concentrations of neoxanthin, violaxanthin and β-carotene was observed. With increasing light intensity, an increase in the concentration of the two xanthophylls neoxanthin and violaxanthin, and β-carotene followed. Appreciable quantities of the three xanthophylls antheraxanthin, zeaxanthin and lutein were biosynthesized only

under the highest light intensity examined here (Figure 8-10). At this light intensity (40 Wm^{-2}) these three compounds appeared in the chromatogram. In addition to the failure to cause formation of these three xanthophylls, the two intermediate light intensities failed to record any significant differences in the amounts of neoxanthin, violaxanthin and β-carotene biosynthesized. The quantities of carotenoids biosynthesized under each light intensity are given in Table 8-3.

8.3.4. Effect of the difference between the daily maximum and minimum temperatures

Figures 8-11 to 8-14 show the effects of the difference between the daily maximum and minimum temperatures on carotenoids biosynthesis. While the xanthophylls experienced large qualitative and quantitative effects of the difference between the maximum and minimum temperatures, the effect on β-carotene was purely quantitative. The amounts of the xanthophylls and the carotene biosynthesized increased with the increasing difference between the maximum and minimum temperatures (up to 8°C) and then failed to record significant increases, or underwent a marginal decrease. The carotenoids content in the leaves transferred from the 'normal' temperature cycle of 25-15°C, to a a difference of 2°C between the maximum and minimum temperatures, suffered a more than 90% decline (Figure 8-11). However, the β-carotene content did not record such a drastic reduction. The other carotenes which normally occurred in traces, recorded an appreciable increase exceeding the quantities of neoxanthin and violaxanthin biosynthesized. However, these carotenes were not quantified. At a temperature difference of 5°C between the day and night temperatures, the biosynthesis of the xanthophylls neoxanthin and violaxanthin recovered somewhat while the β-carotene content did not record an appreciable change quantitatively. The peaks of other carotenes (after β-carotene) did not appear at this level of temperature difference (Figure 8-12). A temperature difference of 8°C induced the most remarkable change in the quantities of all carotenoids biosynthesized. Lutein, the xanthophyll which was not synthesized in significant quantities at the previous levels of temperature differences was present in comparable amounts to the other compounds. The content of violaxanthin also increased by nearly 50%, in comparison with the levels of biosynthesis at 5°C difference between the maximum and minimum temperatures. The biosynthesis of β-carotene increased by 100% (Figure 8-13). Though the biosynthesis of all carotenoids continued at relatively high levels at a difference of 11°C between the maximum and minimum temperatures, the contents of all compounds were less than in the

Figure 8-7: HPLC analysis of the carotenoids of the tea leaves exposed to irradiance of 7 Wm^{-2}

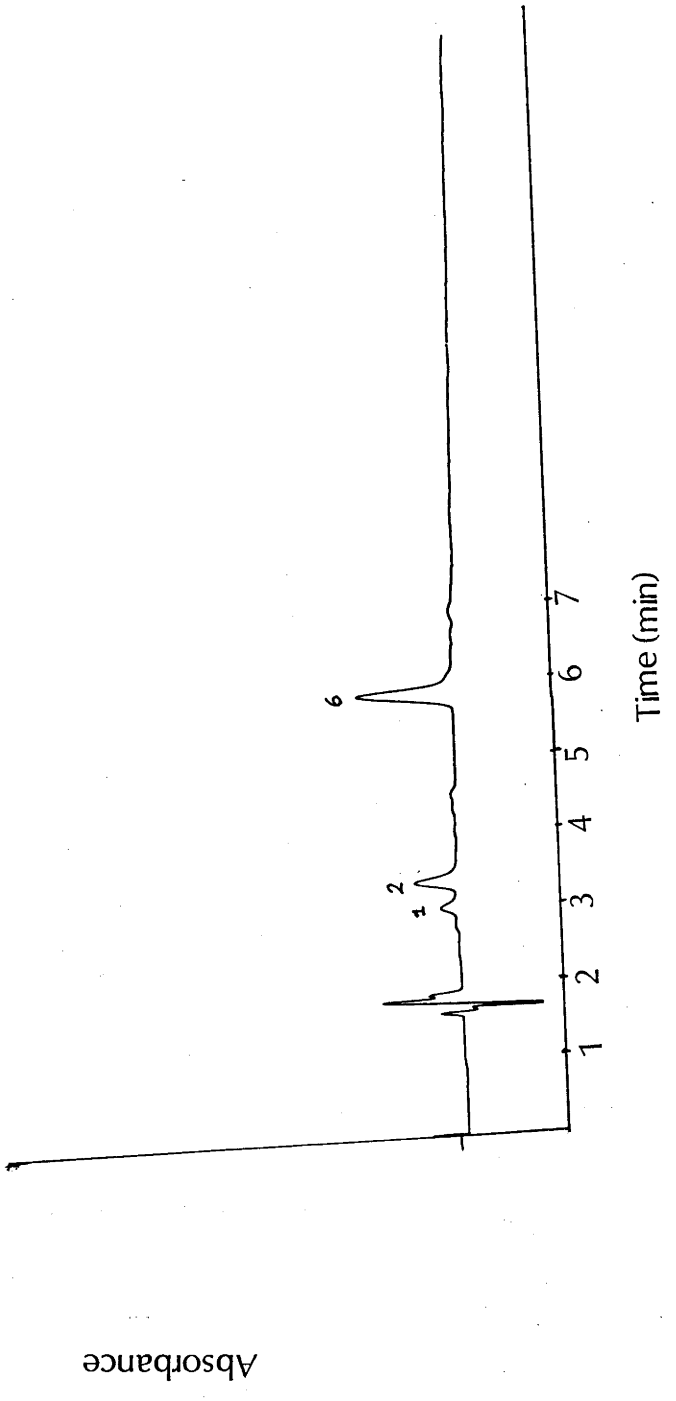
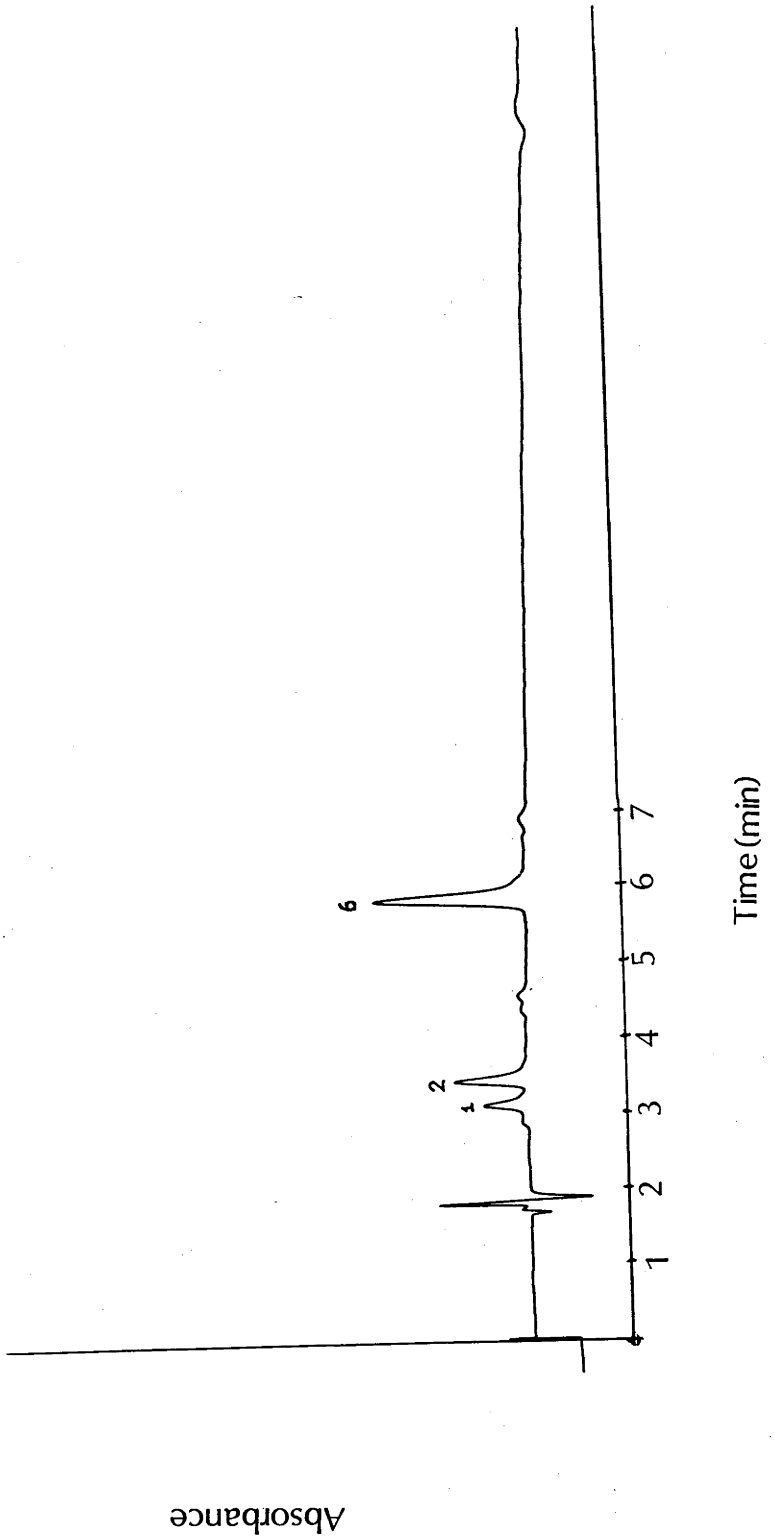

Absorbance
Time (min)
1
2
3
4
5
6
7
1
2
6

Figure 8-8: HPLC analysis of the carotenoids of the tea leaf exposed to irradiance of 15 Wm^{-2}


Absorbance
Time (min)
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1
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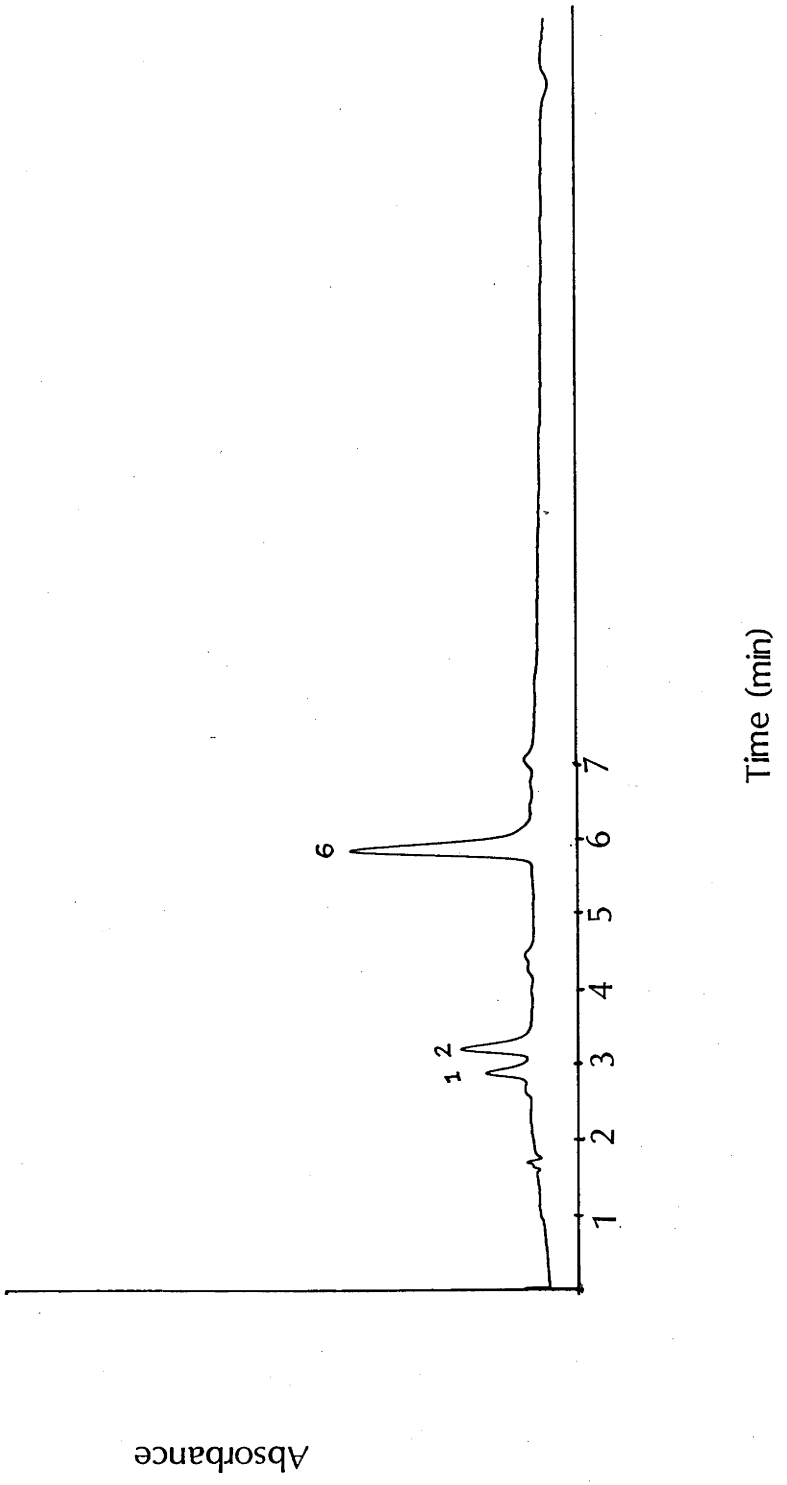
6

Figure 8-9: HPLC analysis of the carotenoids of the tea leaf exposed to irradiance of 30 Wm^{-2}


Absorbance
1
2
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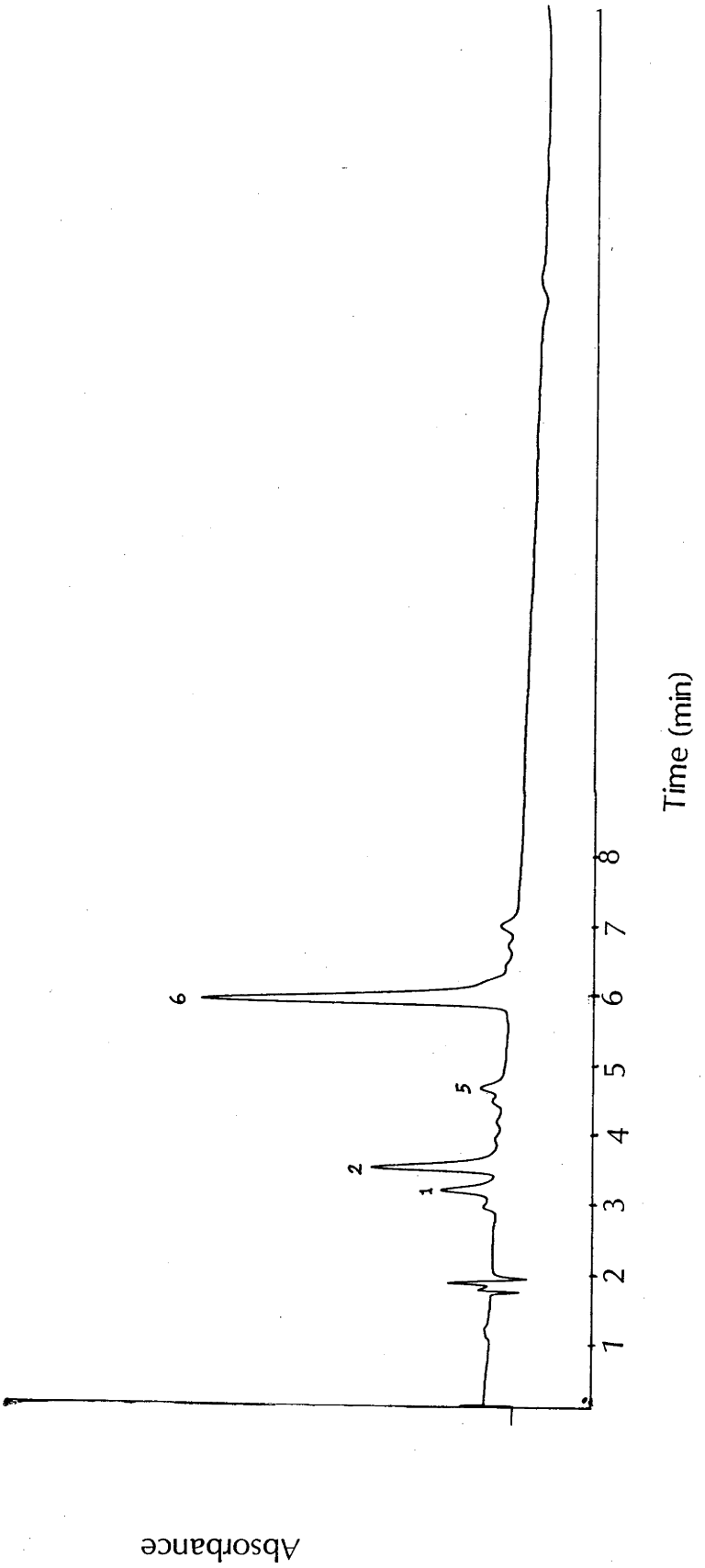
6
7
Time (min)
1
2
6

Figure 8-10: HPLC analysis of the carotenoids of the tea leaf exposed to irradiance of 40 Wm^{-2}


Absorbance
Time (min)
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8
1
2
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	Irradiance (W/m^{-2})				
	0	7	15	30	40
Neoxanthin	-	12.1	15.8	15.4	34.7
Violaxanthin	-	10.8	17.4	18.0	41.3
Lutein	-	-	-	-	8.2
β–Carotene	-	26.7	58.2	64.6	88.4

Table 8-3: Biosynthesis of carotenoids (µg/g dry weight) in the tea leaf under different intensities of irradiance

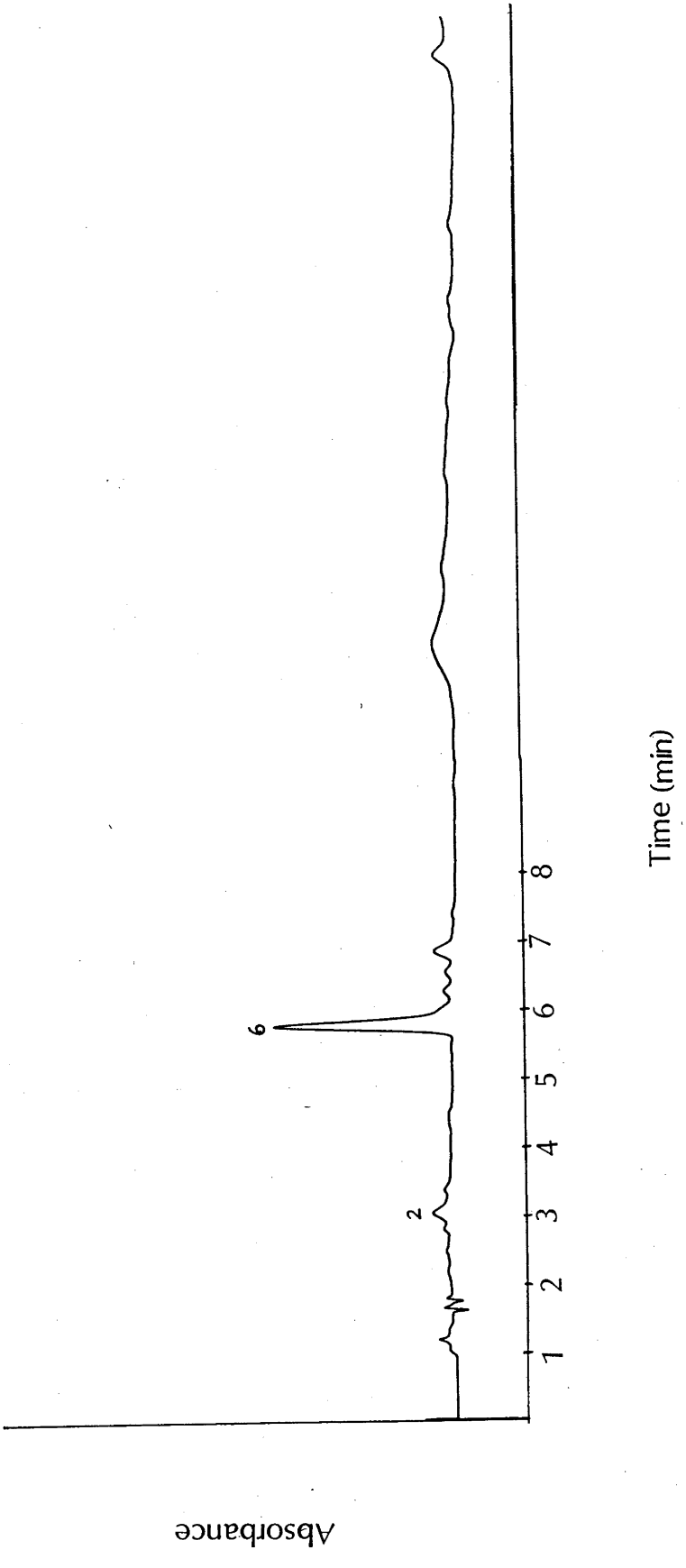
case of 8°C difference. The least effect appeared to be in the case of lutein, which had not undergone a large increase at the earlier level of temperature difference. The other carotenes than β-carotene maintained steady levels through the change in the differences in maximum and minimum temperatures. The quantities of carotenoids biosynthesized under different levels of maximum and minimum temperatures are given in Table 8-4.

8.4. Discussion

The rapid analysis capability of tea leaf carotenoids by rp-HPLC serves several practical needs in the tea industry. In view of the fact that β-carotene enhances tea aroma complex, the measurement of relative contents of this compound in the tea leaf may indicate its quality potential. Such information gathered at harvesting may help in designing the optimum processing conditions essential for the full exploitation of the quality potential of the green tea leaf. At present, the determination of the quality potential of the tea leaf is a subjective process purely based on the weather conditions prevalent at the time of harvest. The content of β-carotene constitutes a quantifiable effect of the weather conditions on the tea leaf and therefore helps in rational formulation of processing conditions. The relatively short analysis time (including sample preparation) enables the analysis of a sample of leaves a day prior to the date of harvesting. An additional application of this test would be to rank newly bred plants according to the quality potential inherent in the plants. The amount of β-carotene biosynthesized by a tea plant under standard environmental conditions would indicate their biosynthetic capacity and therefore, the genetic endowment for the biosynthesis of carotenoids. At present the quality potential of a new tea clone is rated purely on the reputation of its parent plants.

The analysis of tea leaf carotenoids has been performed under similar chromatographic conditions to those employed for the analysis of yeast carotenoids. The conditions determined here have been the optimum for the separation of carotenoid complexes for β-carotene. Resolution of this compound from a variety of other more polar compounds has been successfully attained with a comparatively short retention time. The success of HPLC in separations of carotenoids from such diverse sources demonstrated its versatality. The remarkable reproducibility of retention times further confirmed its reliability. In reversed phase analyses more polar compounds elute before the non polar compounds, in contrast with the conventional HPLC. In the normal phase HPLC analysis of green leaf carotenoids reported by Takagi (1985), β-carotene was the first compound to elute, followed by the xanthophylls. In the present (reversed phase HPLC) analysis, neoxanthin

Figure 8-11: Effect of the difference between the maximum and minimum temperature ($2^{o}C$) on the biosynthesis of carotenoids


Absorbance
Time (min)
1
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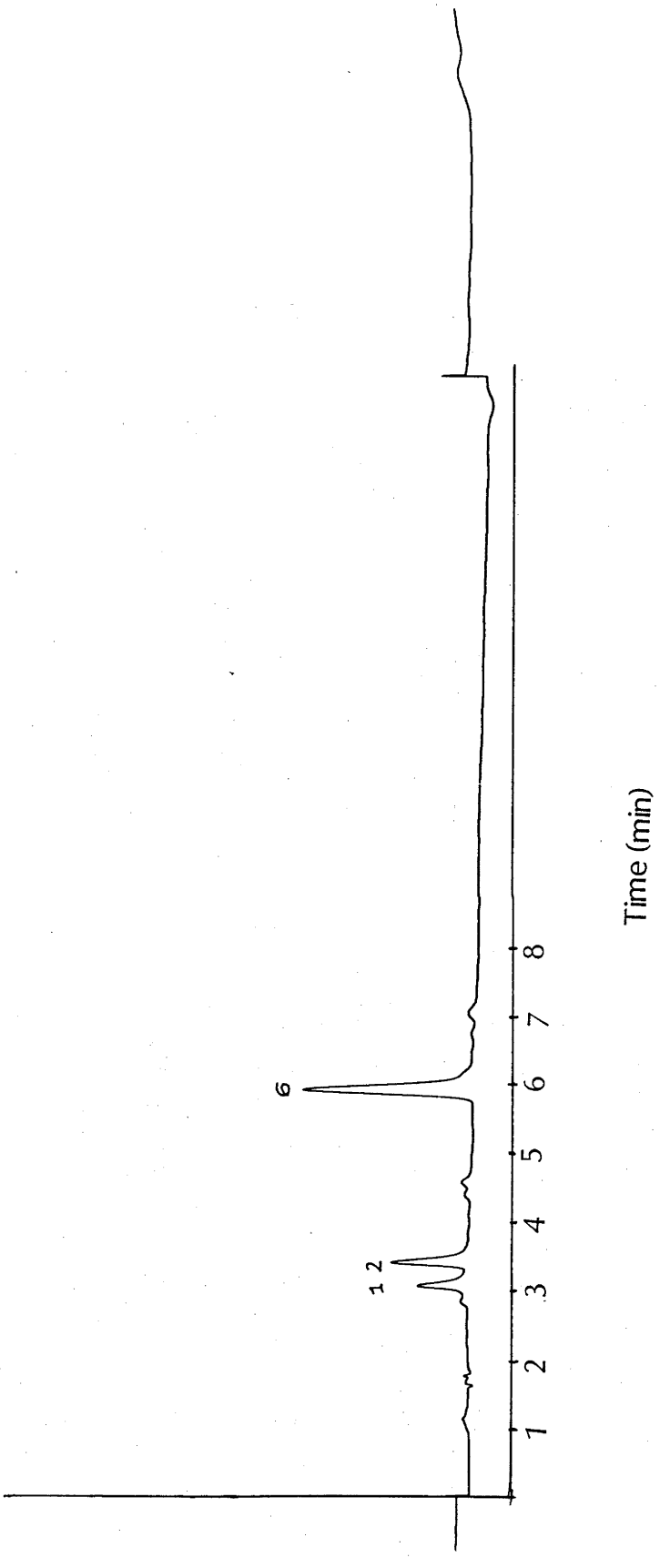
8
2
6

Figure 8-12: Effect of the difference between the maximum and minimum temperature (5°C)on the biosynthesis of carotenoids


Absorbance
Time (min)
1
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1 2
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Figure 8-13: Effect of the difference between the maximum and minimum temperature (8°C) on the biosynthesis of carotenoids

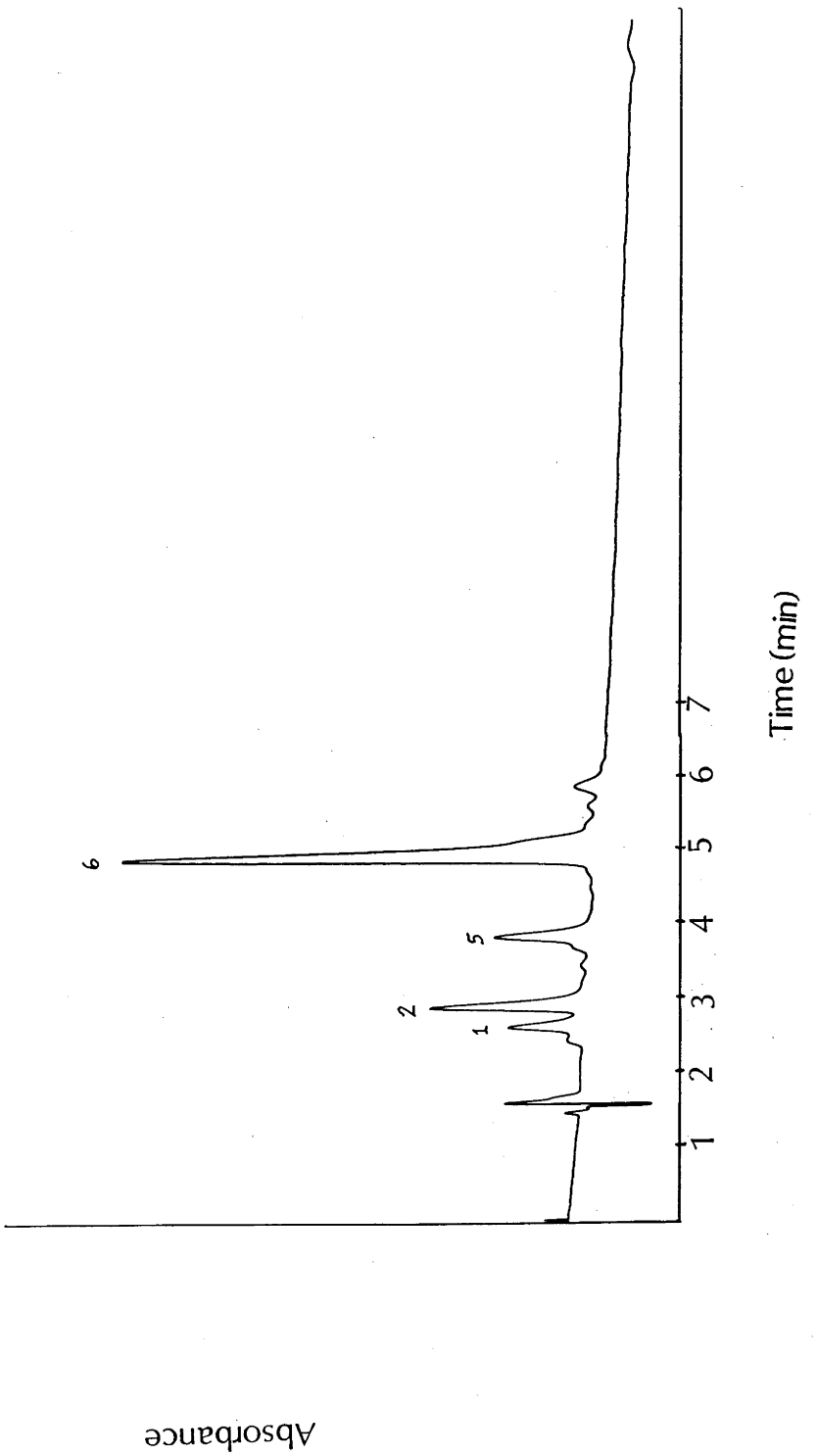
Absorbance
Time (min)
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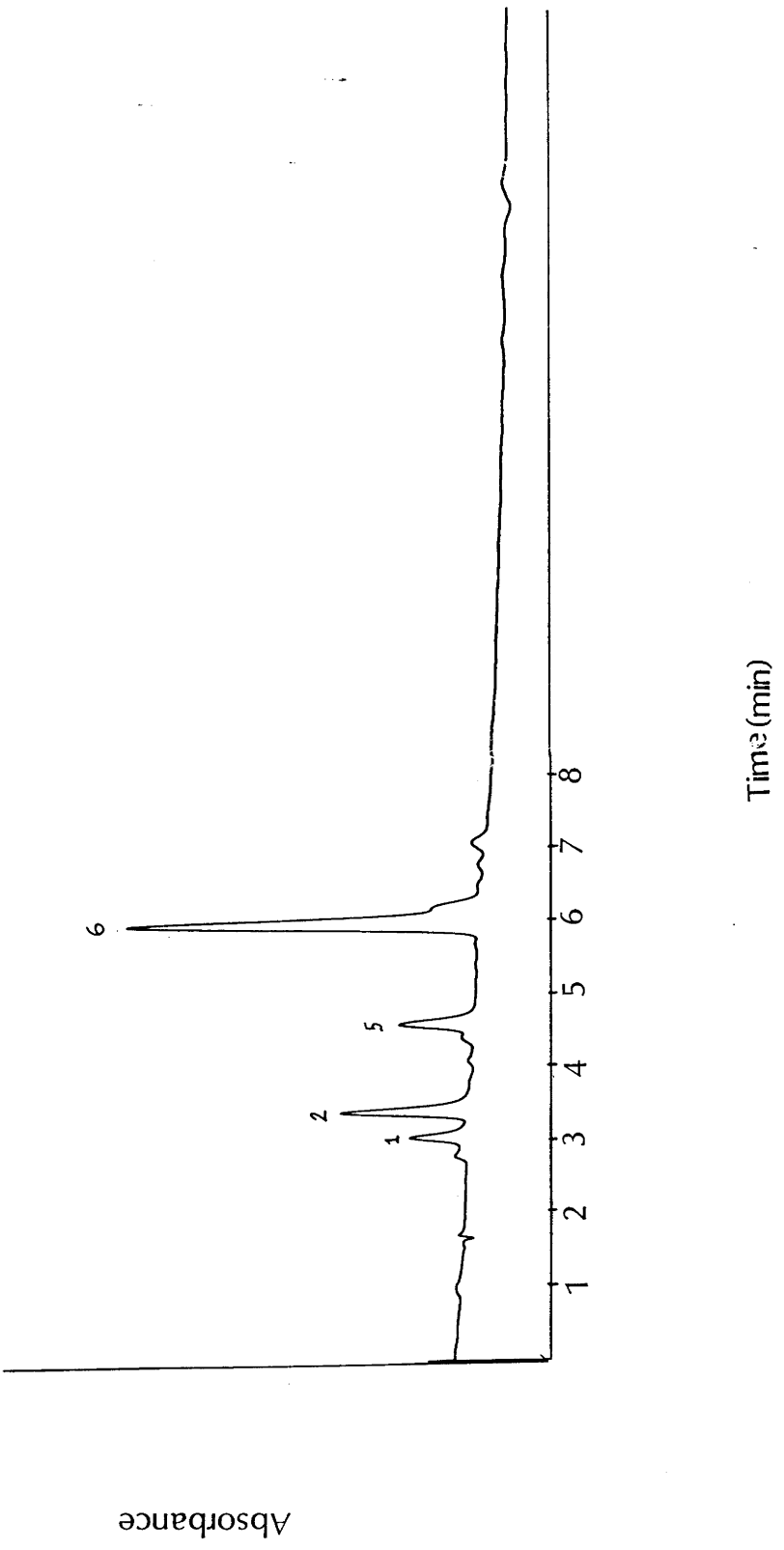
Figure 8-14: Effect of the difference between the maximum and minimum temperature on (11°C) the biosynthesis of carotenoids


Absorbance
Time (min)
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8
1
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6

	Carotenoid content (µg/g dry wt.) Difference between maximum and minimum temperatures			
	2°C	5°C	8°C	11°C
Neoxanthin	-	12.6	22.9	18.1
Violaxanthin	8.4	24.7	46.8	39.4
Lutein	-	-	38.3	17.6
β–Carotene	61.4	64.8	123.3	103.9

ıble 8-4: Biosynthesis of carotenoids under different combinations of maximum and minimum temperatures

eluted first followed by violaxanthin. The last compound to elute was β-carotene. This elution order is based on the relative polarity of the compounds separated. The molecular structure of neoxanthin shows that it contains three hydroxyl (-OH) groups and an epoxy (=O) group in comparison with the two hydroxyl and two epoxy groups contained in the neoxanthin molecule, thus making neoxanthin the more polar. The minor pigments antheraxanthin and zeaxanthin contain two hydroxyl groups each while antheraxanthin gains its higher polarity by the epoxy group contained. The least polar β-carotene was the last compound to elute. The information on molecular structures of the compounds separated serve as corroborative evidence for their relative positions in the chromatograph.

The relative contents of carotenoids of the growing tea shoots of the light-grown tea plants resemble the carotenoid composition of the field grown tea plants. The higher β-carotene content in comparison with the xanthophylls of this leaf sample confirmed the common observation that photosynthetic tissues exposed to light accumulate carotenes whereas those grown under darkness accumulate xanthophylls predominantly. Lichtenthaler (1969) and Wolf (1969) observed a rapid accumulation of β-carotene when plants grown in the dark were exposed to light. Tea leaves in the field during the flavour season in Sri Lanka can thus be expected to synthesize significant amounts of β-carotene.

Analysis of the carotenoids of the bud, and the first and second leaves from shoot apex gave detailed information on the carotenoids content of the unit of harvest in tea. The traditional practice of the use of only the most tender shoot for tea making is based on the fine chemical constitution of these leaves. Analyses of numerous other chemicals including the polyphenols and aroma precursors (Sanderson, 1965; Yamanishi *et al.*, 1969; Sanderson and Sivapalan, 1971) have revealed that the chemical substances that characterize tea as a beverage are concentrated in the bud and the young leaves. The information generated here regarding the carotenoids content in the tender shoot add to this body of knowledge. The remarkable variability exhibited by violaxanthin among the bud and the two leaves probably indicate the interconversions of the xanthophylls and carotenes.

The study of Ullah (1979) contrasts with the results presented here, in that the concentration of β-carotene in the first leaf was more than three times that contained in the bud. Further increases have been recorded from the first to the second leaf. However, the percentage of β-carotene has declined from 49.7% in the bud to 26.2% in the second leaf. In the present experiment, a decline in carotenoids content accompanying leaf senescence

has been observed. The comparison between these two reports show some important differences between the materials used and the methods employed in the analysis. The tea leaves analysed by Ullah (1979) has originated from old, unpruned tea bushes at an elevation of 1500 m in Darjeeling, north India. The plant material used here originated from young seedlings raised in pots in a glass house. The fact that the tea bushes were old and unpruned, suggests a sedate state of physiological activity. Further, being grown in a plantation, they would not have received individual care (in terms of water and nutrition) comparable to the seedlings used in the present experiment. These differences may account for part of the differences in observations. However, the observation that the youngest tissues contain higher amounts of carotenoids is more in accordance with the theory that the bud and the youngest leaves of the tea shoot gives rise to teas with superior flavour. This necessitates critical evaluation of the observed differences between the results of Ullah (1979) and the results presented here. Two important methodological differences that would have influenced the results are apparent. These relate to the preliminary saponification of the pigment extracts and the method of separation involved. Ullah (1979) has adopted 'hot' saponification, during which the total pigments complex, is subjected to temperatures of 50-60°C. The case for 'cold' rather than 'hot' saponification has been argued by Davis (1976). Though time consuming, cold saponification protects thermo-labile carotenoids from structural transformations during this treatment. Secondly, the separation of the carotenoids in the experiment of Ullah (1979) has involved two steps of TLC. Risks of carotenoids undergoing sample loss and structural transformations during TLC have been detailed in Chapter 6. The relative contents of β-carotene, violaxanthin and lutein reported by Ullah (1979) strongly indicate that interconversions of these compounds have taken place during analysis. The inordinate increase in the content of lutein (from 28.5 μg/g dry weight in the bud, to 367.6 and 723.0 μg/g dry weight in the first and second leaves respectively), coupled with a decrease in the contents of violaxanthin and β-carotene bears clear evidence to this effect. Cholonky *et al.* (1958) suggested that β-carotene and zeaxanthin could be converted to the carotenoid epoxide violaxanthin. The conversion of violaxanthin to lutein, upon exposure to light has been demonstrated by Yamamoto *et al.* (1962). This suggests that a conversion of β-carotene to lutein (via violaxanthin) would have been highly probable. The general observation made by Goodwin (1952) that the biosynthesis of carotenoids continues rapidly during the early stages of growth suggests a higher concentration of carotenoids in the growing regions. As maturation progresses there is a marked drop in carotenoids concentration due partly to increased lignification of the tissues and partly to actual loss of carotenes (Goodwin,

1973). Further, the findings of Goodwin (1965) (young and old leaves of maize containing 800 µg/g dry weight and 547 µg dry weight of carotenoids respectively) supports the results presented here. The dramatic reduction in the carotenoids content in mature leaves is probably related to the excessive amounts of chlorophylls contained. The accompanying low concentrations of chlorophylls observed at saponification was a feature of the high carotenoids concentrations in the bud and the two young leaves. Though this observation is inadequate to establish a cause and effect relationship between the high chlorophyll concentrations and low carotenoids concentrations, the common biosynthetic origin of these compounds may have a bearing on the relative concentrations in leaves of different age.

The examination of the effects of light intensity revealed that darkness inhibited the biosynthesis of most carotenoids in the tea leaf, particularly the carotenes. Significant levels of xanthophylls, neoxanthin and violaxanthin were maintained in the dark whereas the levels of β-carotene and the minor xanthophylls were reduced. The total carotenoids complex consisted of a higher percentage of xanthophylls under darkness than under illumination. The treatment levels of irradiance in this experiment (7-40 Wm^{-2}) approximate the field radiation levels. The qualitative and quantitative aspects of carotenoids biosynthesis observed, thus reflect actual situation that could be expected in the field. The results suggest that the relative effect of illumination on the biosynthesis is more on xanthophylls than on β-carotene. However, an accumulation of β-carotene upon exposure to higher light intensities was observed. The relatively higher levels of lutein reported in numerous TLC analyses of the leaf carotenoids may be a result of conversion of other carotenoids to lutein according to the mechanisms referred to above. The difference between the maximum and minimum temperatures exerted similar effects to light intensity on carotenoids biosynthesis. The effect of these factors seem to be higher on the biosynthesis of the xanthophylls. The accumulation of β-carotene seem to be relatively insensitive to these factors.

The study of the effects of light intensity and the difference between maximum and minimum temperatures have revealed that conditions which closely resemble the climatic conditions in Sri Lanka during flavour season, cause qualitative and quantitative changes in the carotenoids biosynthesized. This seems to provide supportive evidence to the hypothesis propounded by Wickremasinghe (1974) that the seasonal occurrence of flavour in tea in Sri Lanka is caused by an increased biosynthesis of carotenoids in the tea leaf during this period. However, a detailed analysis of the carotenoids complex

biosynthesized had not been conducted since the promulgation of that hypothesis. The analyses reveal that an increase in the biosynthesis of numerous components of the carotenoids complex occurs under such conditions. However, detailed experimentation regarding the effect of the increased biosynthesis of the xanthophylls has not been conducted. These compounds too can be expected to contribute to the flavour of tea. The potential contribution of the carotenoids complex of the phylloplane yeasts also had not been forseen by Wickremasinghe (1974). The results presented in this dissertation confirm that an increase in the total carotenoids content of the tea leaf and the yeasts that inhabit the leaf surface occurs during the flavour season in Sri Lanka. However, the events in the tea plant do not offer possibilities of achieving control over the process of flavour biogenesis. The yeast *R. glutinis* offers real possibilities of achieving the long desired objective of inducing flavour in tea.

Chapter 9

General Discussion

The hypothesis that the yeast *Rhodotorula glutinis* enhances tea flavour by supplementing the tea leaf carotenoids is an adjunct to the theory that β-carotene contributes to tea flavour. All theories that have ventured to explain tea flavour have associated β-carotene with this phenomenon. Observations on comparative analyses of flavoury and nonflavory teas enabled by the advent of advanced analytical techniques was chiefly responsible for this orientation. Ever since Tirimanne and Wickremasinghe (1965) first speculated on the association between the tea leaf carotenoids and flavour of black tea, the confirmatory evidence began accumulating. The proof of the contribution made by β-carotene for the flavour of tea was provided by Sanderson *et al.* (1971). They demonstrated with a detailed gas liquid chromatographic analysis of the tea aroma complex that during black tea manufacture β-carotene underwent oxidation to form β-ionone. The significance of this compound as a component of the tea aroma complex had long been known. However, the seasonal nature of tea flavour was not adequately explained until Wickremasinghe (1974) suggested a seasonal increase in the biosynthesis of tea leaf carotenoids. The climatic conditions under which flavour development occurs are characterized by dry, windy, cloudless days followed by cold, clear nights. At least two consecutive weeks of such weather are generally necessary before flavour becomes manifest and even slight rain is known to arrest the development of flavour (Wickremasinghe, 1974). It was postulated that an extra-chloroplastidic pathway of carotenoids biosynthesis from leucine operates in conditions of climatic stress such as those occurring during the flavour season. This theory aroused interest in studies of tea leaf carotenoids and their degradation during tea manufacture. In this purely exploratory research, the possibility of achieving control over the elucidated process of flavour synthesis was neither thought possible nor investigated. It was against this background that the investigations on the alleged role of a 'pink yeast' in flavour development began. The colour of the pigmented cells of the yeast *R. glutinis* indicates its carotenogenic nature. The present research project is conceived on the premiss that the carotenoids of the yeast augment flavour of tea. The experiments, of which the results are presented here, were designed to examine some of the centrally important

conditions for the proposed role of the yeast. These relate to the ability of the yeast to colonise tea leaf surfaces and synthesize β-carotene in this environment under adverse climatic conditions.

By virtue of its concern with a yeast inhabiting the leaf surfaces, this study corresponds to the subject of the microbiology of the phylloplane, an area of research that has been well documented during the last three decades. The interest in phylloplane microbiology emanated primarily from the potential role of the microorganisms colonising leaf surfaces on leaf senescence. The microflora on the surfaces of green leaves that constitute an economic yield (tea, tobacco) deserves special attention due to its implications on the quality of the final product. In the case of tea, the successful growth of the yeast on tea leaf surfaces is sine qua non for the assumed augmentation of tea flavour. Therefore a study of the composition and variations of microbial populations on the leaf surfaces of *Photinia glabra* grown in Canberra was of relevance. In the absence of hard evidence that indicates a host specificity of phylloplane microflora, the observations made here can be regarded as generally applicable. Inadequacies in methods commonly used in the study of phylloplane microorganisms have been discussed by Dickinson (1976). The preliminary comparison of methods carried out here confirmed those fears. However, the methods presently used yield useful results when carefully chosen to study a particular group of microorganisms or a particular aspect of the microflora. The leaf washing technique is suitable for the enumeration of detachable fungal spores on leaf surfaces and the results should be reported accordingly. The use of this method for the study of mycelial growth on leaf surfaces is unacceptable mainly due to its inability to remove representative quantities of fungal mycelia from the leaf surface. Yet this method has often been used inappropriately. The leaf print technique, though incomplete in its recovery of yeasts, yields more consistent data. The quantitative estimations of leaf surface microbial populations thus suffer from a lack of suitable methods of study. However, the results of this study suggest that a reliable picture of the temporal variations of microbial populations could be obtained with the judicious use of available methods.

Differentiation between the microflora on the upper and lower leaf surfaces as done in this study lacks expediency in the context of the ultimate goal of the work presented here. The tea leaf is processed wholly and it is adequate and meaningful to consider the leaf as a system that may either encourage or discourage microbial growth. However, the observations made here on actual differences that exist between the microflora on the two surfaces and particularly the risks of gaining an erroneous impression with regard to

relative microbial activity on either surface of the leaf contributes to the basic knowledge on the phylloplane. It leads to the general conclusion that the information generated by cultural methods needs to be upheld by direct microscopy. The combined use of several cultural methods provide accurate data regarding the composition of the tea leaf surface microflora. However, the simple cataloguing of microorganisms that occur on leaf surfaces is of little theoretical or practical value unless accompanied by information on their activity in this ecological niche. It is abundantly clear that the majority of common aerial contaminants find access to leaf surfaces. Regular sampling of the leaf surface microorganisms is certain to record their presence on leaf surfaces. However, distinguishing the authentic leaf surface inhabitants from numerous casual itinerants is essential for an accurate evaluation of the leaf surface as a habitat. The categorization of microorganisms as potential leaf surface inhabitants should be preceded by the determination of activity (signified by growth) on the leaf surface. Direct microscopy is indispensable for this purpose.

The correlation between meteorological parameters and leaf surface microbial populations constitutes a highly relevant piece of information for an explanation of the climatically induced phenomenon of tea flavour on the basis of microbial activity. The necessity of specific climatic conditions for the development of flavour and increased biosynthesis of carotenoids in the tea leaf under such conditions has been observed (Wickremasinghe, 1974; Hazarika and Mahanta, 1983). The proof of ability of the yeast to grow and biosynthesize carotenoids under those climatic conditions is fundamental for the assumed role. However, the environmental conditions that induce tea flavour are generally known to cause stress in the tea plant (leaf). These conditions can in general be expected to inhibit microbial growth. As revealed by the results of the study of the phylloplane of *P. glabra*, the populations of leaf surface microflora are positively correlated with the total rainfall and average relative humidity during the fortnight prior to the date of sampling. This effect could be consequent to the effect on the composition of aerial microflora which in turn reflects sporulation of fungi elsewhere. The acceptance of the hypothesis propounded here demands explanations of how the yeast *R. glutinis* could be an exception to this generality. The key to its success may lie in pigmentation. Last and Warren (1972) suggested that the pigmented microorganisms are most successful on leaf surfaces. During bright, sunny weather the pigmentation of yeast cells bestows upon it a competitive advantage over nonpigmented organisms that may succumb to this combination of hostile conditions. In such a situation the yeast encounters less competition for the available water

on the leaf surface. Higher rates of transpiration during such weather maintains high humidities in the boundary layer, thus countering the effects of harsh environmental conditions. The positive effect of the difference between the maximum and minimum temperatures on the growth of phylloplane microorganisms suggests that dew formed under such conditions (characteristic of the flavour season) provides an extra source of water. It is established that exudation of nutrients on to the leaf surfaces is increased when the plants are under stress (Tuckey, 1971). The combination of above conditions should be conducive for the substantial growth and multiplication of the yeast. Confirmatory evidence for such growth was obtained in the study of tea leaf surface microflora. The unexpected discovery of a tea enterprise in north Queensland enabled a compositional analysis of the tea leaf surface microflora, though in a study marred by distance and lack of laboratory facilities. The 'dry season' in tropical north Queensland resembles the climatic conditions in the tea growing regions of Sri Lanka during the flavour season. The enumeration of the tea leaf surface microflora occurring under these climatic conditions revealed that the yeast *R. glutinis* is a major constituent. In addition to the yeasts a multitude of mycelial fungi which are common aerial contaminants were encountered on tea leaf surfaces. Differences between the composition of the leaf surface microflora in north Queensland and under mild temperate conditions in Canberra can be attributed to the differences between the aerial microflora in the two locations. Even though the degree of activity of these fungi could not be determined, it could be safely assumed that the majority occurred as inactive spores.

The results of these studies of the phylloplane imply that the majority of fungi that find access to leaf surfaces fail to colonise this environment. Availability of water had earlier been identified as the factor that limit microbial growth on leaf surfaces (Roger and Blakeman, 1975). This observation prompted the examination of the effects of water stress on spore germination and mycelial growth of fungi commonly reported on the phylloplane. The investigation of the spore germination and mycelial growth of three common mycelial fungi under low osmotic water potentials yielded a partial explanation of the inability of these organisms to colonise leaf surfaces. Studies of water relations of phylloplane fungi have been conducted on experimental systems in which the fungus was not in close contact with the controlling water potential. The fungi tested in this experiment were placed on water potential amended culture medium, thus better subjecting the fungi to the water potential of the substrate. However, the important factor of microbial interactions that influences water relations of fungi on leaf surfaces is not incorporated in to such

experimental systems as the one employed here. This aspect warrants consideration in the interpretation of the results. The variety of ionic and nonionic solutes (salts and sugars) used in this experiment allowed examination of the effect of the solute on fungal growth response to low water potentials. The results confirmed the common observation that the solute used to obtain water potential exerts a nutritive or toxic effect at low and high concentrations respectively. However, in an experiment designed to test the effects of a range of water potentials the actual effect of water potential becomes clearly visible.

Even though the fungal spores under examination failed to germinate at water potentials below -4 MPa, their growth on the leaf surface cannot be totally discounted. The strategy adopted by these fungi may be to remain dormant during the adverse environmental conditions and resume growth when the conditions are more favourable. Thus their success may be determined more by the reserves available for utilisation through long periods of dormancy rather than the ability to achieve germination under adverse environmental conditions. This accounts for the incompatibility sometimes observed between the performance of fungi in experimental systems and the growth on the leaf surface. However, the germinated spore may be more vulnerable to the vagaries of environmental factors. The mycelial growth response of the fungi in water potential amended culture medium will therefore indicate better their actual performance on leaf surfaces.

Leaf water potentials are unlikely to be much lower than the critical water potentials necessary for growth of these fungi. In fact, the water potential at leaf surfaces are reported to be marginally higher than in the tissues (Burrage, 1971). The desiccating effect of bright, warm and windy conditions may aggravate the problem of limited availability of water. Resistance to ultra violet radiation in the intense solar radiation during this period may be the critical factor which determines the success of a microorganism on the leaf surface. The pigmented microorganisms and microbial propagules thus command a distinct advantage over the nonpigmented forms. The extraordinary success of the pigmented yeasts *R. glutinis* and *Sporobolomyces roseus* has been attributed to their being pigmented (Last and Warren, 1972). The apparent success of the spores of *Epicoccum nigrum* (which were commonly found on leaf surfaces in Canberra) also could be attributed to their thick, melanized spore walls which can resist the damaging effect of radiation. Lack of special adaptations to achieve germination and mycelial growth under low water potentials seem to hinder the establishment of the majority of fungi that occur on leaf surfaces. In the absence of compensatory traits such as pigmentation of cells the opportunities for these

fungi to succeed in this inhospitable environment appear to be rare. However the majority of these spores exhibit a capacity to withstand adverse weather conditions and resume growth when favourable conditions recur.

The information generated in the experiments designed to investigate the ability of the yeast *R. glutinis* to maintain growth and the biosynthesis of carotenoids under limited availability of water are centrally important for the proof of the hypothesis propounded here. The climatic conditions that induce water stress in the tea plant are commonplace during the tea flavour season in Sri Lanka, and are essential for flavour synthesis. The growth and carotenoids production by the yeast under low water potential showed similar trends. Processes of growth and carotenoids biosynthesis declined beyond moderately low water potentials of 3-4 MPa. Growth ceased at around -10 MPa. The cessation of carotenoid biosynthesis at a somewhat higher water potential than the critical water potential for growth suggests that pigment biosynthesis occurs only after successful growth has been achieved. Diversion of limited resources of energy for the primary metabolic process of growth may be responsible for this lag phase before the biosynthesis of pigments begins. When the growth is adversely affected the additional demands on energy may be responsible for the retardation of pigments biosynthesis. The effect of the age of culture on carotenoids biosynthesis (qualitative and quantitative) suggests the operation of a pathway similar to the one proposed by Simpson (1972). According to this pathway γ-carotene once biosynthesized may convert either to β-carotene or torulene, which later transforms to torularhodin. Increasing age of culture seems to accelerate the conversion of γ-carotene to torularhodin, thus reducing the biosynthesis of β-carotene. The increased conversion of γ-carotene with age may be caused either by the accumulation of an enzyme responsible, or by the accumulation of γ-carotene. The effect of illumination on carotenoids biosynthesis appear to be simpler. It seems to trigger off the process of carotenoids biosynthesis and upon commencement the process appears to continue irrespective of the conditions of illumination. The fact that the biosynthesis of carotenoids in yeasts undergoes qualitative and quantitative changes in response to cultural conditions augurs well for its future industrial application. This indicates that the potential exists for increasing the content of β-carotene in preference to other components of the carotenoids complex by variating the cultural conditions.

The investigation of the effects of cultural conditions on the biosynthesis of the total carotenoids complex provides important basic knowledge on the subject. However, it does not yield information regarding the biosynthesis of β-carotene, the study of which

compound required separation of the pigments complex. Commonly used methods of separation have been often criticized on the grounds that these methods expose the extremely labile carotenoids to the atmosphere causing transformation, decomposition and artefact formation. Incomplete sample recovery in TLC is a further disadvantage. Despite these justifiable criticisms open column chromatography and TLC have been the methods conventionally employed in the separation of carotenoids. Possible changes the extremely labile structures of the carotenoids could undergo during separation have not received critical evaluation. High pressure liquid chromatography (HPLC) is a sensitive, rapid and more accurate method of analysis particularly suited for the separation of carotenoids. This method protects the labile structures of carotenoids from exposure to atmospheric oxygen and light which causes structural transformations and artefact formation. The short analysis time HPLC affords is a distinct advantage in experiments involving large numbers of analyses. However, the weakly polar nature of the carotenoids necessitated the use of reversed phase stationary phases for their separation. The use of the C_{18} stationary phase solves the problems of long retention times in octadecylsilane (ODS) columns and inadequate resolution in C_8 columns. Though the development of suitable conditions of HPLC for carotenoids analysis consumed a considerable length of time this application is certain to recompense in the long run in terms of time saved in comparisons of the quality potential of different tea clones. The analysis of the yeast carotenoids using HPLC adds to the increasing application of this technique in carotenoids separations. Its earlier application in the area of microbial carotenoids was limited to the separation of the carotenoids of the smut fungus *Ustilago violacea*. The carotenoids of this fungus consisting only of carotenes makes the analysis more conventional and easier to perform than in the case of yeast carotenoids which include a mixture of nonpolar carotenes and the polar xanthophylls torulene and torularhodin. Inclusion of compounds of a wide range of polarities in the same chromatograph necessitated compromise in retention time for β-carotene. However, the increased precision of analysis more than compensated for the slight lengthening of retention time which was still considerably shorter than the analysis times achievable with any of the conventional techniques.

The effects of carbon and nitrogen nutrition, age of cells and conditions of illumination and temperature on the biosynthesis of β-carotene and torularhodin further confirm the biosynthetic pathway suggested by Simpson (1972). The relative synthesis of these two compounds under the conditions tested in this experiment clearly suggests that β-carotene is an intermediate compound in the process, the formation of which depends on the

suitability of conditions for the conversion of γ-carotene to β-carotene. Under conditions dissimilar to these γ-carotene seems to be converting to torulene and torularhodin predominantly. The observed beneficial effects of the yeast on the flavour of tea suggests that the combination of environmental conditions during the flavour season diverts the biosynthesis along the pathway to β-carotene. Under adequate availability of nutrition and water temperature could be the only factor that could possibly be detrimental for this diversion. However, the windy conditions prevailing during the flavour season may be exerting a moderating influence on temperature. The effect of light appear to be inconsequential for the biosynthesis of yeast carotenoids under such bright conditions existing in the field during the flavour season. The observed detrimental effect of rainfall on flavour could be explained on the basis of its effect on the biosynthesis of tea leaf carotenoids. Higher cell water contents in the leaf may be activating the normal photosynthetic pathway in preference to the pathway elucidated by Wickremasinghe (1974). Light may have an important effect on the biosynthesis of carotenoids in the tea leaf unlike in the case of the yeast.

The carotenoid contents of the bud, the first, second and third leaves provide an additional explanation for the historical practice of utilising only the most tender shoots of the tea plant for the production of the beverage. All chemical analyses hitherto performed have revealed that chemical compounds that impart a fine flavour to tea are concentrated in the growing part of the shoot. The compounds known to accumulate in the growing parts of the tea plant include the polyphenols, terpenoids and as revealed by this study, the carotenoids. The maturing of the shoot has been observed to accompany a decline in the concentration of these compounds. The concomitant increase in the chlorophyll content with the decrease in the carotenoids content in mature tea leaves indicates a diversion of the biosynthetic pathways from the other terpenoids to chlorophylls. The qualitative differences in the carotenoids too confirm such a diversion. The effect of the increased biosynthesis of lutein and violaxanthin may not be totally detrimental to the quality of tea since these compounds are also converted to volatile compounds that enrich tea aroma. The increased chlorophyll content however may detract from flavour while strengthening the blackness of processed tea. The trends of β-carotene biosynthesis under temperature and light conditions similar to the flavour season seems to be consistent with the hypothesis of Wickremasinghe (1974). Under more intense light conditions and wide differences between the day and night temperatures a significant increase in β-carotene biosynthesis occurs. This confirms the widely held belief that β-carotene is associated with flavour.

The pattern of carotenoid biosynthesis observed shows that the total carotenoids content increases in addition to the increase in the biosynthesis of β-carotene. However when the above hypothesis was propounded the supplementary role played by the yeast carotenoids was unknown.

Even though this study concerns the subsidiary role played by the yeast carotenoids in tea flavour synthesis, the tea leaf carotenoids occupy primary importance in any conceptual study of tea flavour. The study of the biosynthesis of carotenoids in the tea leaf employing HPLC constitutes an important development in this respect. The application of HPLC in the separation of tea leaf carotenoids provides a rapid method of predicting the quality potential of a particular leaf sample being processed, or a tea clone commissioned for field cultivation. The carotenoids content of the tea leaf provides an additional criterion of the quality potential. While conditions of growth of the plants for such tests need to be controlled the prediction of the quality potential of a particular leaf sample serves a more practical marketing need. Tea processing conditions are generally decided according to the quality potential of the green leaf and the final quality of the made tea desired. This involves subjective determination of the quality potential of the leaf. The rapid analysis of a representative sample of leaf may give a better indication of the quality contained in the leaf thus affording a better opportunity of determining optimum processing conditions for the production of best quality.

9.1. Thoughts for the future and directions for future research

9.1.1. Immediate future

The results reported here lend support to the proposition that the pink yeast *R. glutinis* colonising tea leaf surfaces during the tea flavour season in Sri Lanka enhances flavour of tea by supplementing the tea leaf carotenoids. The beneficial role of β-carotene in tea aroma formation has been confirmed earlier. The ultimate proof of the proposed effect of the yeast on tea aroma awaits processing of tea leaf supplemented with different levels of yeast populations. The beneficial effect of the yeast could be demonstrated by adding yeast suspensions on to tea leaf surfaces before processing and comparing the aroma complex of the resultant tea with that of uninoculated teas. The findings suggest that the industrial usage of the yeast to induce flavour in tea during the 'nonflavoury' weather conditions is feasible. Such a process which incorporates a yeast (similar to the processes of wine making and brewing of beer) adds a new dimension to the age old practice of tea making.

This converts the tea manufacturing process which is hitherto considered as a biochemical process, to a biotechnological process.

Practical considerations of the culturing and application of the yeast on to tea leaf surfaces need careful further study. Initially a scaling-up of the β-carotene production by the yeast has to be devised as usually done in biotechnological applications of microorganisms. The commercially available fermentors would facilitate this process. The methods of culturing the yeast and incorporating onto the leaf surfaces that involve least renovations to the existing processing plant and equipment have to be sought.

Tea manufacturing programmes usually involve batch processing, requiring batch fermenters of suitable capacity for the culture of yeasts. A shift from the solid culture medium to aerated broth is necessary to facilitate spraying of the yeast. The fermenters will need to be placed at a higher elevation in the processing plant and the cultures drawn into a network of pipes fitted with sprinklers after separating yeast cells from the broth. The sprinklers should be placed above the withering troughs and the yeast sprayed at predetermined intervals with mixing of leaves in the trough. The introduction of the yeast at the withering stage is critically important due to its mode of conversion to the aroma compounds. Sanderson (1971) observed that the β-carotene to β-ionone conversion occurred only in the presence of oxidised polyphenols. The introduction of the yeast broth at fermentation will increase drying time and will increase costs of processing. Introduction in the withering troughs will allow excess moisture to be evaporated by the forced draught and allow the yeasts to coat the leaf surface. During the leaf rolling operation this layer will be thoroughly mixed with the polyphenols exuded from the leaf cells and provide optimum conditions for the chemical conversion.

9.1.2. Medium to long term research needs

Upon determination of the practicalities of the process of tea manufacture with the yeast the path taken by the comparable processes needs to be followed. This includes strain improvement of the yeast to maximise β-carotene biosynthesis. As revealed by the results here the manipulation of cultural conditions may provide limited opportunities of increasing the yield of β-carotene biosynthesized by the yeast. However, this may prove inadequate in the long run due to random genetic variations that may occur in yeast populations. Extreme strain purity and specialisation has been a feature of traditional industries exploiting yeasts. Modern techniques in molecular biology and recombinant

DNA technology offer ample opportunities of achieving such strain improvement. A continuous programme of monitoring variations in the capacity of the yeast to biosynthesize carotenoids and introduction of new improved strains will be necessary.

Appendix A

Composition of the basal medium used in water relations experiments

Na_2HPO_4	0.75 g
KH_2PO_4	0.75 g
$MgSO_4$	0.12 g
NaCl	0.10 g
NH_4NO_3	0.40 g
Glucose	1.80 g
Yeast extract	1.0 g
Malt extract	1.0 g
Agar	15 g
H_2O	1 L
pH	6.4
Water potential	-0.12 MPa

(Adapted from Sommers *et al.*, 1970)

Appendix B

Molal-based dry weights of solutes used for characterizing water relations of phylloplane fungi

	Solute (g/1000g H_2O)			
WP*(MPa)	Glucose	Sucrose	KCl	Na_2SO_4
-1	72.1	134.2	16.6	25.3
-2	144.2	260.1	33.5	54.1
-3	214.1	377.9	50.4	84.7
-4	280.7	489.2	67.2	116.8
-5	344.7	595.1	84.0	150.0
-6	420.0	696.3	100.5	183.0
-7	487.0	791.4	116.7	215.1
-8	553.0	886.4	132.9	247.1
-10	685.0	1066.0	164.5	306.0

Appendix C

Chromatograph of the test mixture RP-D

DESCRIPTION SERIAL NO: 051685-1-4
PACKING MATERIAL: ALLTECH C18 5U
LENGTH(MM): 250 ID(MM): 4.6
TEST CONDITIONS
MOBILE PHASE: 65/35 ACETONITRILE/WATER
FLOW RATE: 1
DETECTOR: UV 254NM

CATALOG NO: 605RPC

FITTING CODE: C

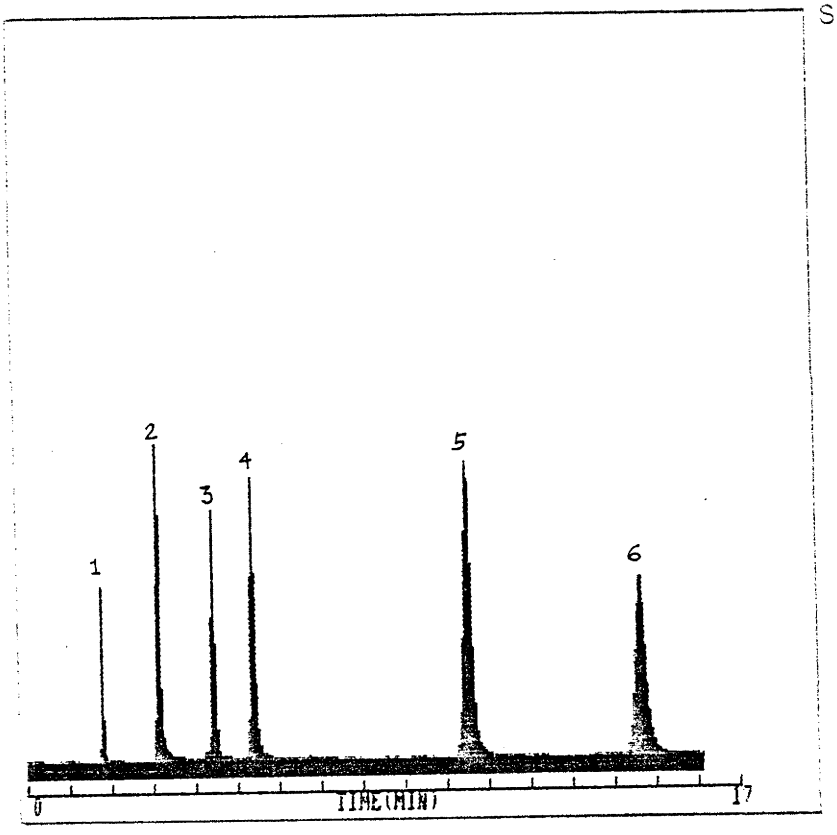
PRESSURE(PSIG): 1600
SENSITIVITY: .5

SAMPLE

RPMIXD

COMPONENT

1 URACIL
2 PHENOL
3 BENZALDEHYDE
4 N-N-DIET-M-TOLUAMIDE
5 TOLUENE
6 ET BENZENE



CALCULATIONS

PEAK NO	RETENTION TIME(MIN)	CAPACITY FACTOR	SYMMETRY	EFFICIENCY (PLATES/M)
1	2.04	0.02	1.47	48259.55
2	3.25	0.63	1.85	41611.55
3	4.43	1.22	1.31	53031.57
4	5.28	1.64	1.61	219173.42
5	9.9	3.95	1.38	54867.58
6	13.62	5.81	1.33	55586.94

ALLTECH ASSOCIATES

QUALITY ASSURANCE CHROMATOGRAM

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